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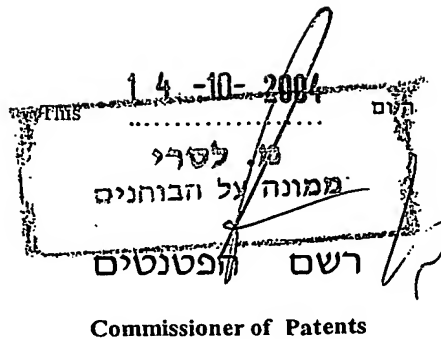
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wner, : Assignment

העברה

בעל אמצאה מכח  
by virtue of

invention the title of which

(Hebrew)

נוגדנים ל NIK, הכנתם והשימוש בהם

ששמה הוא

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Antibodies to NIK, their preparation and use

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# Antibodies to NIK, their preparation and use

נוגדנים ל-NIK, הכנתם והשימוש בהם

Yeda Research and Development Co. Ltd.

Ref: IL/924

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## ANTI-NIK ANTIBODIES AND USES THEREOF

5

### FIELD OF THE INVENTION

The present invention relates to immunoregulatory molecules. More particularly, the present invention relates to antibodies or antibody fragments capable of specifically binding NF- $\kappa$ B-inducing kinase (NIK)/MAP3K14, or a specific portion thereof, and of thereby regulating a biochemical activity of NIK, and/or enabling  
10 detection of NIK or a specific portion thereof.

### BACKGROUND OF THE INVENTION

No treatment or no satisfactory treatment exists for numerous lethal and/or  
15 highly debilitating diseases associated with deregulated activity of NF- $\kappa$ B molecules, including malignant diseases and diseases associated with pathological immune responses, such as autoimmune, allergic, inflammatory, and transplantation-related diseases.

NF- $\kappa$ B family molecules are eukaryotic transcription factor complexes critical  
20 for the regulation of immune responses, cell growth, and survival (Ghosh S. *et al.*, 1998. *Annu Rev Immunol.* 16:225-60) which are inducible activated by almost all TNF/NGF receptor family members. NF- $\kappa$ B molecules are normally sequestered in the cytoplasmic compartment by physical association with a family of cytoplasmic ankyrin-rich inhibitors termed I $\kappa$ B, including I $\kappa$ B $\alpha$  and related proteins (Baldwin AS.  
25 Jr., 1996. *Annu Rev Immunol.* 14:649-83). In response to diverse stimuli, including cytokines, mitogens, and certain viral gene products, I $\kappa$ B is rapidly phosphorylated at Ser32 and Ser36, and is ubiquitinated and subsequently degraded by the 26S proteasome. This allows the liberated NF- $\kappa$ B to translocate to the nucleus and participate in target gene transactivation (Mercurio F. and Manning A.M., 1999. *Curr Opin Cell Biol.* 11:226-32; Pahl, H.L., 1999. *Oncogene* 18:6853-66). Recent  
30 molecular cloning studies have identified a multi-subunit I $\kappa$ B kinase (IKK) complex that mediates the signal-induced phosphorylation of I $\kappa$ B, an inhibitor of NF- $\kappa$ B. The IKK complex is composed of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory

subunit IKK $\gamma$  (NEMO). The catalytic activity of both IKK $\alpha$  and IKK $\beta$  can be activated by a multitude of different NF- $\kappa$ B inducers, including inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1, the T-cell receptor (TCR) and the T-cell costimulatory protein, CD28 (Karin, M. and Ben-Neriah, Y., 2000. *Annu Rev Immunol.* 18:621-63).

NF- $\kappa$ B-inducing kinase (NIK)/MAP3K-14 (WIPO Pub. No. WO9737016A1 to the present inventors) is critical for activation of NF- $\kappa$ B. For example, overexpression of NIK has been shown to lead to dramatic activation of NF- $\kappa$ B (reviewed in Wallach D. *et al.*, 2002. *Arthritis Res.* 4 Suppl 3:S189-96), and expression of catalytically-inactive NIK mutants has been shown to lead to effective inhibition of NF- $\kappa$ B activation in response to a variety of known NF- $\kappa$ B activators, such as LMP1, TNF receptor (TNFR)-1, TNFR-2, RANK, human Toll receptor, CD3/CD28, IL-1 receptor (IL-1R), human T-cell lymphotropic virus (HTLV)-1 Tax protein, and lipopolysaccharide (LPS) (Malinin, N.L. *et al.*, 1997. *Nature* 385:540-4; Sylla, B.S. *et al.*, 1998. *Proc Natl Acad Sci U S A.* 95:10106-11; Darnay, B.G. *et al.*, 1999. *J Biol Chem.* 274:7724-31; Lin, X. *et al.*, 1999. *Immunity* 10:271-80; Geleziunas, R. *et al.*, 1998. *Mol Cell Biol.* 18:5157-65). Targeted disruption of the NIK gene (Yin, L. *et al.*, 2001. *Science* 291:2162-5), and study of the 'alymphoplasia' (aly) mouse strain bearing a natural Gly855Arg missense point mutation in NIK (Shinkura, R. *et al.*, 1999. *Nat Genet.* 22:74-7) revealed that NIK has an essential role in lymphoid organ development. Both aly/aly and NIK knockout mice manifest systemic absence of lymph nodes and Peyer's patches, disorganized splenic and thymic architectures, and immunodeficiency whose most resilient features are low serum immunoglobulin levels and lack of graft rejection (Shinkura, R. *et al.*, 1999. *Nat Genet.* 22:74-7). These abnormalities apparently reflect aberrant signaling by a variety of receptors. The developmental deficiencies of the NIK mutant mice resemble those found in mice deficient in the LT- $\beta$  receptor (LT- $\beta$ R) suggesting that NIK also participates in signaling by this particular receptor. Impaired B cell proliferative capacity in the aly/aly mice could be shown to correlate to a deficient response of these cells to LPS and CD40 ligand (CD40L; Garceau, N. *et al.*, 2000. *J Exp Med.* 191:381-6), and presence of excessive amounts of B1 cells in the peritoneal cavity of mice could be ascribed to defects in homing of peritoneal cells to the gut-associated lymphatic tissue

(GALT) system as a consequence of deficient chemokine receptor signaling in secondary lymphoid tissue (Fagarasan, S. *et al.*, 2000. *J Exp Med.* 191:1477-86).

An important and general role for NIK in cytokine receptor signaling has been recently demonstrated in studies performed by Wallach *et al.* who have shown, using a two-hybrid system, that the IL-2 receptor  $\gamma$  chain, or "common  $\gamma$  chain" which is a signaling component the receptors for IL-2, -4, -7, IL-9, -13, -15 and -21, specifically associates with NIK (PCT/IL 03/00317). Overexpression of the common  $\gamma$  chain was found to potentiate NF- $\kappa$ B activation mediated by NIK, and upon IL-2 or IL-15 stimulation, NIK and signalosome components were found to bind to common  $\gamma$  chain. These results therefore indicate involvement of NIK in signaling via the large variety of cytokine receptors comprising common  $\gamma$  chain as a signaling subunit.

Apart from these and other contributions to the regulation of the development and function of the immune system, NIK is also involved in the regulation of various non-immune functions. The aly/aly (though not the NIK knockout) mice display deficient mammary gland development (Miyawaki, S. *et al.*, 1994. *Eur J Immunol.* 24:429-34). Moreover, *in-vitro* studies have implicated NIK in signaling leading to skeletal muscle cell differentiation (Canicio, J. *et al.*, 2001. *J Biol Chem.* 276:20228-33), and to survival and differentiation of neurons (Foehr, E.D. *et al.*, 2000. *J Biol Chem.* 275:34021-4).

Consistent with the suggested role of NIK as mediator of NF- $\kappa$ B activation, fibroblasts derived from aly/aly and NIK knock-out mice fail to activate NF- $\kappa$ B in response to LT- $\beta$ R activation. Moreover, LT- $\beta$ R upregulation of VCAM-1, which occurs through NF- $\kappa$ B activation, is abnormal in aly/aly mouse embryonic fibroblasts (MEFs; Matsumoto, M. *et al.*, 1999. *J Immunol.* 163:1584-91). Deficient phosphorylation of I $\kappa$ B has also been noted in the response of aly/aly B-lymphocytes to CD40 ligation. In contrast, in dendritic cells of these mice, CD40-induced phosphorylation of I $\kappa$ B appeared normal (Garceau, N. *et al.*, 2000. *J Exp Med.* 191:381-6). Aly/aly peritoneal cells are also incapable of responding to the chemokine SLC with increased NF- $\kappa$ B activity (Fagarasan, S. *et al.*, 2000. *J Exp Med.* 191:1477-86).

Assessment of the pattern of expressed NF- $\kappa$ B species in lymphoid organs of aly/aly mice indicated that, apart from its role in the regulation of NF- $\kappa$ B complex(es)

comprised of Rel proteins (A+p50) and I $\kappa$ B, NIK also participates in controlling the expression/activation of other NF- $\kappa$ B species. Most notably, lymphocytes of *aly/aly* are deficient for p52, an NF- $\kappa$ B species that is specifically formed in mature B lymphocytes through proteolytic processing of an inactive precursor, p100 (NF- $\kappa$ B2), suggesting a deficiency in p100 to p52 conversion (Yamada, T. *et al.*, 2000. J Immunol. 165:804-12). Indeed, NIK has been shown to participate in site-specific phosphorylation of p100. Both directly lead to phosphorylation of IKK $\alpha$  which in turn phosphorylates p100. This phosphorylation serves as a molecular trigger for ubiquitination and active processing of p100 to form p52. This p100 processing activity was found to be ablated by the *aly* mutation (Xiao, G. *et al.*, 2001. Mol Cell 7:401-9; Senftleben, U. *et al.*, 2001. Science 293:1495-9).

In view of the structural homology of NIK to MAP3Ks, some attempts have been made to explore the involvement of NIK in the ERK, JNK and p38 cascades, the three other main protein kinase cascades involving MAP3Ks (Akiba, H. *et al.*, 1998. J Biol Chem. 273:13353-8). NIK has been shown to be involved in the ERK cascade in PC12 pheochromocytoma cells (Foehr, E.D. *et al.*, 2000. J Biol Chem. 275:34021-4). Evidence has also been presented that in certain cells NIK may participate in signaling for phosphorylation of Jun, the downstream target of the JNK cascade, independently of this particular cascade (Akiba, H. *et al.*, 1998. J Biol Chem. 273:13353-8; Natoli, G. *et al.*, 1997. J Biol Chem. 272:26079-82). Overall, these findings indicate that NIK indeed serves as a mediator of NF- $\kappa$ B activation, but may also serve other functions, and that it exerts these functions in a cell- and receptor-specific manner.

Similarly to other MAP3Ks, NIK can be activated as a consequence of phosphorylation of the 'activation loop' within the NIK molecule. Indeed, mutation of a phosphorylation-site within this loop (Thr559) prevents activation of NF- $\kappa$ B upon NIK overexpression (Lin, X. *et al.*, 1999. Immunity 10:271-80). In addition, the activity of NIK seems to be regulated through the ability of the regions upstream and downstream of its kinase motif to bind to each other (Lin *et al.* Molec. Cell Biol. (18) 10 5899-5907 1998). The C-terminal region of NIK downstream of its kinase moiety has been shown to be capable of binding directly to IKK $\alpha$  (Regnier, C.H. *et al.*, 1997. Cell 90:373-83) as well as to p100 (Xiao, G. and Sun, S.C., 2000. J Biol Chem. 275:21081-5) and to TRAF2 (Malinin, N.L. *et al.*, 1997. Nature 385:540-4). Such

interactions are apparently required for NIK function in NF- $\kappa$ B signaling. The N-terminal region of NIK contains a negative-regulatory domain (NRD), which is composed of a basic motif (BR) and a proline-rich repeat motif (PRR) (Xiao, G. *et al.*, 2001. *Mol Cell* 7:401-9). Apparently, the N-terminal NRD interacts with the C-terminal region of NIK in cis, thereby inhibiting the binding of NIK to its substrate (IKK $\alpha$  and p100). Ectopically expressed NIK seems to spontaneously form oligomers in which these bindings of the N-terminal to the C-terminal regions in each NIK molecule are apparently disrupted, and display a high level of constitutive activity (Lin, X. *et al.*, 1999. *Immunity* 10:271-80). The binding of the NIK C-terminal region to the TNF-receptor-associated adaptor protein TRAF2, as well as to other TRAFs, (Malinin, N.L. *et al.*, 1997. *Nature* 385:540-4; Rothe, M. *et al.*, 1994. *Cell* 78:681-92; Takeuchi, M. *et al.*, 1996. *J Biol Chem.* 271:19935-42) most likely participates in the activation of NF- $\kappa$ B by NIK.

Evidence has been presented that NIK, through the binding of its C-terminal region to IKK $\alpha$ , can activate the IKK complex. It has been shown to be capable of phosphorylating Ser176 in the activation loop of IKK $\alpha$  and to thereby activate this molecule (Ling, L. *et al.*, 1998. *Proc Natl Acad Sci U S A.* 95:3792-7). Consistently with such a mode of action, studies regarding the mechanisms accounting for the deficiency in NF- $\kappa$ B activation by LT- $\beta$ R in aly/aly MEFs have indicated that the NIK mutation ablates activation of the IKK signalosome and the consequent phosphorylation of I $\kappa$ B (Matsushima, A. *et al.*, 2001. *J Exp Med.* 193:631-6). The ability of NIK to bind p100 directly through its C-terminal region and phosphorylate it suggests that p100 serves as a direct NIK substrate (Xiao, G. and Sun, S.C., 2000. *J Biol Chem.* 275:21081-5). Nevertheless, a recent study has suggested that NIK mediates p100 phosphorylation in an indirect way, through phosphorylation and thus activation of IKK $\alpha$  that in turn phosphorylates p100 (Senftleben, U. *et al.*, 2001. *Science* 293:1495-9).

Thus, activation of NF- $\kappa$ B molecules by NIK represents a critical control point for regulation of NF- $\kappa$ B activity.

As described above, deregulated NF- $\kappa$ B activity is associated with the pathogenesis of various major human diseases, such as numerous malignant diseases and diseases associated with pathological immune responses (reviewed in Yamamoto



and Gaynor, 2001. *J Clin Invest.* 107:135-142). For example, activation of the NF- $\kappa$ B pathway is prominently involved in the pathogenesis of chronic inflammatory disease, such as asthma, and rheumatoid arthritis (Tak and Firestein, 2001. *J Clin Invest.* 107:7-11; Karin, M. and Ben-Neriah, Y., 2000. *Annu Rev Immunol.* 18:621-63), and inflammatory bowel disease. In addition, altered NF- $\kappa$ B regulation appears to be involved in the pathogenesis of other diseases, such as arteriosclerosis (Collins and Cybulsky, 2001. *J Clin Invest.* 107:255-64; Leonard, W.J. *et al.*, 1995. *Immunol Rev.* 148:97-114) and Alzheimer's disease (Mattson and Camandola, 2001. *J Clin Invest.* 107:247-54; Lin, X. *et al.*, 1999. *Immunity* 10:271-80), in which the inflammatory response is at least partially involved.

Several lines of evidence indicate that NF- $\kappa$ B activation of cytokine genes is an important contributor to the pathogenesis of asthma, which is characterized by the infiltration of inflammatory cells and the deregulation of many cytokines and chemokines in the lung (Ling, L. *et al.*, 1998. *Proc Natl Acad Sci U S A.* 95:3792-7). Cytokines, such as TNF, that activate NF- $\kappa$ B are elevated in the synovial fluid of patients with rheumatoid arthritis and contribute to the chronic inflammatory changes and synovial hyperplasia seen in the joints of these patients (Malinin, N.L. *et al.*, 1997. *Nature* 385:540-4). The administration of antibodies directed against TNF or a truncated TNF receptor that binds to TNF can markedly improve the symptoms of patients with rheumatoid arthritis.

Increases in the production of proinflammatory cytokines by both lymphocytes and macrophages have also been implicated in the pathogenesis of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis (Matsumoto, M. *et al.*, 1999. *J Immunol.* 163:1584-91). NF- $\kappa$ B activation is seen in mucosal biopsy specimens from patients with active Crohn's disease and ulcerative colitis. Treatment of patients suffering from inflammatory bowel diseases with steroids decreases NF- $\kappa$ B activity in biopsy specimens and reduces clinical symptoms. These results indicate that stimulation of the NF- $\kappa$ B pathway is involved in the enhanced inflammatory response associated with these diseases.

Atherosclerosis is triggered by numerous insults to the endothelium and smooth muscle of the damaged vessel wall (Matsushima *et al.*, 2001). A large number of growth factors, cytokines, and chemokines released from endothelial cells, smooth

muscle, macrophages, and lymphocytes are involved in this chronic inflammatory and fibroproliferative process (Matsushima, A. *et al.*, 2001. *J Exp Med.* 193:631-6). NF- $\kappa$ B regulation of genes involved in the inflammatory response and in the control of cellular proliferation is widely understood as playing an important role in the initiation and progression of atherosclerosis.

As described above, abnormalities in the regulation of the NF- $\kappa$ B pathway have been shown to be involved in the pathogenesis of Alzheimer's disease. For example, NF- $\kappa$ B immunoreactivity is found predominantly in and around early neuritic plaque types in Alzheimer's disease, whereas mature plaque types show vastly reduced NF- $\kappa$ B activity (Mercurio F. and Manning A.M., 1999. *Curr Opin Cell Biol.* 11:226-32). Thus, NF- $\kappa$ B activation is in the initiation of neuritic plaques and neuronal apoptosis during the early phases of Alzheimer's disease. These data therefore indicate that activation of the NF- $\kappa$ B pathway plays a role in a number of diseases that have an inflammatory component involved in their pathogenesis.

In addition to a role in the pathogenesis of diseases associated with deregulated immune responses, hyper-activation or constitutive activation of the NF- $\kappa$ B pathway has also been implicated in the pathogenesis of various human cancers. Abnormally high and/or constitutive activation of the NF- $\kappa$ B pathway is frequently seen in a variety of human malignancies including leukemias, lymphomas, and solid tumors (Miyawaki, S. *et al.*, 1994. *Eur J Immunol.* 24:429-34). These abnormalities result in abnormally and/or constitutively high levels of NF- $\kappa$ B in the nucleus of a variety of tumors including breast, ovarian, prostate, and colon cancers. The majority of these changes are likely due to alterations in regulatory proteins that activate signaling pathways that lead to activation of the NF- $\kappa$ B pathway. However, mutations that inactivate I $\kappa$ B proteins, in addition to amplification and rearrangements of genes encoding NF- $\kappa$ B family members, can result in the enhanced nuclear levels of NF- $\kappa$ B seen in some tumors (Emmerich *et al.* *Blood* Nov 1;94 (9):3129-34, 1999).

Since, as described above, NIK is critical for activation of NF- $\kappa$ B, one potentially potent strategy for regulating activation of NF- $\kappa$ B, and thereby for treating diseases associated with deregulated NF- $\kappa$ B activity, involves identifying antibodies capable of specifically binding NIK or a specific portion thereof, and of thereby preventing or inhibiting activation of NF- $\kappa$ B by NIK.

Prior art approaches have failed to provide antibodies capable of binding NIK phosphorylated at amino acid T559. One such antibody, which was supposed to be specific to NIK phosphorylated at T559, was purchased last year (2002) from Santa Cruz (SC12957R). However the antibody did not work properly in our hands, and this year the antibody was removed from the catalogue.

Currently, a method of detecting different molecular species of NIK comprising phosphorylated active and non-phosphorylated species employs overexpression in cells of epitope-tagged NIK and anti-tag antibody. This method of detection of phosphorylated NIK has many limitations one of them is that the method cannot be used to specifically detect endogenous activated NIK.

There is thus a widely recognized need for, and it would be highly advantageous to have, antibodies capable of binding endogenous phosphorylated NIK.

#### SUMMARY OF THE INVENTION

The invention relates to an antibody or fragment thereof e.g. wherein said antibody fragment is selected from the group consisting of a single-chain Fv, an Fab, an Fab', an F(ab')<sub>2</sub>, and a CDR being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof such as that in SEQ ID NO 6 and 3, wherein the amino acid sequence or portion thereof comprises a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5. The antibody of the invention can be an IgG antibody, polyclonal, monoclonal, e.g. antibodies generated by hybridoma NIK-P4 30.12 deposited at the CNCM under No. I-3095.

The antibody according to the invention is further capable of regulating a biochemical activity of a NIK molecule and of specifically detecting phosphorylated NIK or a specific portion thereof e.g. by being used in Western blot analysis, ELISA and or immunoprecipitation.

In another aspect, the invention provides monoclonal antibodies generated by hybridoma NIK-P4 30.12 deposited at the CNCM under No. I-3095.

In addition, the invention relates to a pharmaceutical composition comprising a

pharmaceutically acceptable carrier and, as an active ingredient, being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5. More specifically the pharmaceutical composition comprising an antibody or antibody fragment which is further capable of regulating a biochemical activity of a NIK molecule, e.g. capable of inhibiting signalling of CD40 and/or CD70.

The invention provides also, a method of regulating a biochemical activity of a NIK molecule, the method comprising contacting the NIK molecule with being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5, thereby regulating a biochemical activity of a NIK molecule. More specifically said contacting of the NIK molecule with said preparation can be effected by administering said preparation to an individual.

In one embodiment the invention relates to a composition-of-matter comprising a substrate, such as an affinity chromatography matrix, covalently attached to a peptide of amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof e.g. that of SEQ ID NO:3, wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 for selectively capturing the antibody or antibody fragment capable of specifically binding the target antigen.

In another embodiment, the invention relates to a method of treatment of a disease caused or aggravated by the activity of NIK, comprising the administration of an antibody being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof

comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 to an individual in need.

In addition, the invention teaches the use of an antibody or antibody fragment being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 in the manufacture of a medicament for the treatment of a disease caused or aggravated by the activity of NIK.

Also the invention provides a method for preparing a monoclonal antibody comprising growing a cloned hybridoma comprising a spleen cell from a mammal immunized with an amino acid sequence comprising SEQ ID NO: 6, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 and a homogeneous or heterogeneous lymphoid cell in liquid medium or mammalian abdomen to allow the hybridoma to produce and accumulate the monoclonal antibody.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 shows a diagram depicting NIK domains, T559, the N- terminal region the kinase region, and the C-terminal domain of NIK (405-420), etc.

FIG. 2 shows a sequence depicting the amino acid sequence of human NIK (SEQ ID NO: 5). NIK-derived peptide used for immunization SEQ ID NO:3 is underlined.

Figure 3 shows the amino acid sequence of the activation loop of NIK (SEQ ID NO: 4)

Figure 4 shows immunoprecipitation (IP) with different monoclonal antibodies prepared using phosphorylated peptide set forth in SEQ ID NO:3.

Cells were transfected with pCS3MTNIK (WT-NIK) or, with the vector encoding unphosphorylatable version of the protein, pCS3MTNIKT559A (NIK-). Transfected cells were harvested and lysed in 2ml 1% NP-40 buffer. 100ul of this lysate was used for each IP. IP was carried out with protein G beads adsorbed with the indicated antibodies. Western blotting detection and positive control for IP was performed with anti-myc antibody.

Fig 5 shows differential binding of antibody prepared with peptide of SEQ ID NO:3 to phosphorylated peptide derived from the activation loop by ELISA. Control and phosphorylated peptide derived from the activation loop peptide (SEQ ID NO:3) were coated in microtiter plates at a concentration of 10µg/ml. Undiluted hybridoma culture supernatant of clone NIK-P4 30.12 was added for 1 hour at 37°C Anti mouse HRP was used as the secondary antibody at 1:10K dilution and O.D 405 was measured using ABTS substrate

Fig 6 shows the effect of NIK-P4 30.12 on IκB degradation induced by CD70, CD40 or TNF. Ramos cells were transfected with IgG (negative control) or NIK-P4 30.12 antibody (α-pNIK). The transfected cells remained untreated or were treated with CD70, CD40 and TNF for 20 min and 30min and 20 min. respectively. Following ligand treatment the cells were lysed and subjected to Western blotting detected with anti IκB antibody.

Fig 7 shows the effect of NIK-P4 30.12 vis-avis a different antibody developed against a peptide of NIK located also in the kinase domain, but outside of the activation loop, on I $\kappa$ B degradation induced by CD40.

5 Cells were transfected with IgG (negative control ), NIK-P4 30.12 antibody (p4) or a different antibody developed against a peptide of NIK located also in the kinase domain, but outside of the activation loop (SEQ ID NO:7, 81). The transfected cells remained untreated (-cd40) or were treated with CD40 (+cd40) for 30 min. Following ligand treatment the cells were lysed and subjected to Western blotting detected with  
10 anti I $\kappa$ B antibody.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to an antibody or antibody fragment capable of  
15 specifically binding NF- $\kappa$ B-inducing kinase (NIK)/MAP3K14 (SEQ ID NO:5) phosphorylated at residue T559 or to a specific portion thereof. Specifically, the antibody of the present invention can be used for regulating a biochemical activity of NIK, and for specifically detecting NIK phosphorylated at residue T559 or a specific  
20 portion thereof. By virtue of enabling such regulation and such detection, the antibody can be used, respectively, for regulating NF- $\kappa$ B activation, and hence for treating a disease associated with deregulated NF- $\kappa$ B activity, and for characterizing aspects of normal/pathological biological/biochemical processes/states involving NIK or a specific portion thereof.

25 Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of  
30 description and should not be regarded as limiting.

No treatment, or no satisfactory treatment, is available for numerous lethal and/or highly debilitating diseases associated with deregulated NF- $\kappa$ B activity. Such

diseases include malignant diseases and diseases, such as autoimmune, allergic, inflammatory, transplantation-related diseases, which are associated with pathological immune responses. Since NIK is a critical activator of NF- $\kappa$ B, one potentially potent strategy for treating a disease associated with deregulated NF- $\kappa$ B activity involves identifying antibodies capable of specifically binding phosphorylated NIK or a specific portion thereof, and using such antibodies for therapeutically regulating NF- $\kappa$ B activity. By virtue of enabling specific detection of phosphorylated NIK or a specific portion thereof, such antibodies would also enable characterization of aspects of normal/pathological biological/biochemical processes/states involving phosphorylated NIK or a specific portion thereof.

While reducing the present invention to practice, an antibody was unexpectedly generated capable of: specifically binding or optimally specifically binding NIK (SEQ ID NO:5) phosphorylated at T559, or a specific portion thereof such as that set forth in SEQ ID NO:3. The capacity of the antibody of the present invention to specifically bind NIK phosphorylated at T559, or to a portion thereof such as the portion set forth in SEQ ID NO:3 is unique relative to all prior art antibodies.

Hence, in sharp contrast to prior art antibodies, the antibody of the present invention can be used for optimally regulating a biochemical activity, such as a kinase activity of NIK or a specific portion thereof, and for optimally detecting NIK phosphorylated at T559 or for a specific portion thereof. The antibody of the invention can also be used to co-immunoprecipitate and isolate regulatory factors binding to NIK phosphorylated at T559.

It will be appreciated by the ordinarily skilled artisan that an antibody preparation such as the one of the present invention can be treated using standard methods, for example as described herein below, so as to generate a preparation of one or more types of antibody fragment having antigen-binding characteristics essentially identical as those of the untreated antibody preparation.

Thus, according to one aspect of the present invention, there is provided an antibody or antibody fragment capable of specifically binding NIK phosphorylated at T559, or a portion thereof such as the amino acid sequence set forth in SEQ ID NO: 3



The term “a portion of a peptide” is defined as at least a tripeptide within the activation loop wherein one out of the amino acids is phosphorylated threonine and the adjacent amino acids at the N terminus and C-terminus are glycine and glutamic acid respectively i.e. GT(p)E.

- 5 Portions of phosphorylated activation loop (SEQ ID NO: 4 ) having as the penultimate amino acid the phosphorylated T599 is referred to hereinafter as “target antigen”. T599 is the threonine residue at position T599 in the whole NIK protein (SEQ ID NO:5) and is also the threonine residue which is conserved in the derived portions thereof. E.g. Threonine at position 11 in SEQ ID NO:3, Threonine at position 26 in  
10 SEQ ID NO: 4, Threonine at position 26 in SEQ ID NO:6.

- As described herein below, the antibody can be used for optimally regulating the biochemical activity of NF-κB inducing kinase (NIK)/MAP3K14, such as a kinase activity, and hence can be used for optimally regulating activation of NF-κB, and thereby can be used for optimally treating a disease associated with deregulation of  
15 NF-κB activity. Furthermore, the antibody can be uniquely used for detecting the phosphorylated activation loop region of NIK, or any of various specific portions thereof, and can be used for optimally detecting any of various specific portions of the activation loop region of NIK. As such antibody can be utilized for optimally characterizing aspects of normal/pathological biological/biochemical processes/states  
20 involving NIK phosphorylated at T559 or specific portions thereof.

As used herein, the term “treating” when relating to a disease refers to preventing onset of a disease, alleviating a disease, attenuating or eliminating the symptoms of a disease, slowing or reversing the progression of a disease, or curing a disease.

- 25 As used herein, the term “antibody” refers to a substantially whole or intact antibody molecule.

As used herein, the phrase “antibody fragment” refers to a molecule comprising a portion of an antibody capable of specifically binding an antigen, an antigenic determinant or an epitope.

- 30 As described in the Examples section, below:

The amino acid sequence set forth in SEQ ID NO: 3, 4, and 6 represents amino acid residues 549-560, 534-566 and 534-560 of human NIK, respectively, and are

located in the activation loop of human NIK;

Preferably, the antibody of the invention is capable of binding the target antigen with maximal affinity.

- 5 The antibodies of the invention e.g. antibodies having binding capacity to the target antigen according to the invention, are capable of specifically and efficiently detect NIK phosphorylated at T559 by Western blot analysis.

- 10 The antibodies of the invention e.g. antibodies having binding capacity to the target antigen according to the invention, are capable of specifically and efficiently detect NIK phosphorylated at T559 by ELISA.

- 15 The antibodies of the invention e.g. antibodies having binding capacity to the target antigen according to the invention, are capable of specifically and efficiently immunoprecipitate NIK phosphorylated at T559.

- 20 In a specific embodiment polyclonal and monoclonal antibodies specific for phosphorylated NIK, were generated using as the immunizing peptide 549-560 (SEQ ID NO:3) of amino acid sequence S L L T G D Y I P G T(p) E in which T(p) is phosphorylated threonine and is located in the penultimate amino acid of the sequence.

- 25 Also, additional peptides derived from the activation loop of amino acid sequence DFGHAVCLQPDGLG K S L L T G D Y I P G T(p) E (SEQ ID NO:6 ) or portion thereof can be used as the immunizing peptide provided that the phosphorylated T559 is located at the penultimate amino acid in the sequence.

- Also additional peptides comprising the activation loop amino acid sequence DFGHAVCLQPDGLG K S L L T G D Y I P G T(p) E (SEQ ID NO:6 ) or portion thereof can be used as the immunizing peptide provided that the phosphorylated T559 is located at the penultimate amino acid in the sequence.

- 30 Alternatively a cysteine a lysine or any other amino acid can be chemically fused to the N-terminal end to the immunizing peptide.

The term "specifically bind " according to the invention relates to antibodies that can

bind to NIK phosphorylated at T559, but in contrast, they have low binding capacity, or do not bind at all to non phosphorylated NIK. The differential binding of the antibody of the invention can be tested by different assays e.g. by ELISA, Western blot hybridisation and immunoprecipitation.

5 For generation of monoclonal antibody according to the invention different species of immunized mice can be used, preferably of the SJL strain. Also, different hematopoietic organs of the immunized mouse can be used for fusion to generate the hybridoma e.g. spleen and /or lymph node.

10 The antibodies prepared according to the invention are capable of recognizing the entire NIK protein or fragments thereof in the phosphorylated state.

In another embodiment it has been demonstrated that the antibodies according to the invention can inhibit degradation of I $\kappa$ B and consequently activation of NF $\kappa$ -B induced by CD40 or CD70 but not by TNF ligand. These results show that the antibody of the  
15 invention can inhibit specifically activation of NF $\kappa$ -B.

Therefore, the antibody according to the invention can be used for therapy in diseases in which CD70 and CD40 signalling is involved in the pathogenesis of the disease.

20 The inhibition of NIK activation observed with the antibody of the invention e.g. NIK-P4 30.12, does not occur with other antibodies specific for NIK capable of binding domains in the kinase which exclude the activation loop region of NIK.

25 In general, an antibody of the present invention capable of binding the target antigen with maximal affinity will enable optimal down-regulation of a biochemical activity mediated or associated with the target antigen. Similarly, a preparation of the present antibody capable of specifically binding the target antigen with maximal affinity will generally enable detection of the target antigen with optimal sensitivity.

30 By virtue of the above-described capacity of the preparation for specifically binding a target antigen of the present invention, in particular by virtue of the unique capacity of the preparation for specifically binding a target antigen of the present

invention located in a functional region of NIK, the preparation can be used for down-regulating a biochemical activity of NIK. In particular, by virtue of the above-described unique capacity of the antibody for specifically binding a target antigen of the present invention located in the activation loop region of NIK, the preparation can be used for optimally down-regulating the activity of NIK. It will be appreciated that since such kinase activity is crucial for activating nuclear factor (NF)- $\kappa$ B, as described above, an antibody of the present invention capable of optimally down-regulating such kinase activity can be used for optimally down-regulating activity of NF- $\kappa$ B.

The NF- $\kappa$ B proteins is a family of proteins that share a 300 amino acid domain, the "Rel homology domain". The Rel homology domain mediates the DNA binding, dimerization, and nuclear transport of NF- $\kappa$ B proteins. In addition to the Rel homology domain, some members of the NF- $\kappa$ B family also contain a transactivation domain(e.g. c-Rel, RelB, and p65). The NF- $\kappa$ B members p50 and p52, are produced upon activation by lysis of inactive precursors p105 and p100, respectively. P50 and p52c have DNA binding dimerization properties but not strong transactivation domains. It is the differential generation of these proteins, their ability to heterodimerize with different family members, and the interaction of these proteins with different components of the transcription apparatus that contribute to the diverse effects of activating the NF- $\kappa$ B pathway. NIK does not affect all NF- $\kappa$ B species but only specific NF- $\kappa$ B species e.g. it induces degradation of p100 and generation of p52c, in response to specific inducers e.g. lymphotoxin. Modulation of NIK, e.g. using the antibodies of the invention, is therefore likely to affect specific aspects of the pathological implications NF- $\kappa$ B activation. This is an advantage since general non-specific inhibition of NF- $\kappa$ B is dangerous, for example, it can result in extensive apoptosis in the liver.

As used herein the phrase "down-regulating" when relating to a biochemical activity refers to preventing, reducing, or inhibiting such a biochemical activity.

When employed for inhibiting a biochemical activity of NIK, the antibody is preferably capable of specifically binding a maximal number of target antigens within a portion of NIK associated with such biochemical activity. In particular, when employed for inhibiting kinase activity of NIK, the antibody is preferably capable of

specifically binding a maximal number of target antigens located within the NIK activation loop region amino acid sequences set forth in SEQ ID 3 and 4. While a preparation of the present invention capable of specifically binding only one target antigen located within a functional region of NIK, such as the activation loop region, may be employed for efficiently inhibiting a biochemical function of NIK, such as kinase activity, associated with such a functional region, an antibody capable of specifically binding a maximal number of target antigens located within such a functional region will more effectively down-regulate the kinase activity by virtue of interfering with a maximal number of functional epitopes within the functional region.

Alternately, for inhibiting kinase activity of NIK, the antibody may be advantageously capable of specifically binding a maximal number of target antigens located within the amino acid sequences set forth in SEQ ID NO 3 and 4 and 6.

By virtue of the above-described capacity of the antibody for specifically binding a target antigen of the present invention, the preparation can be used for specifically detecting NIK phosphorylated at T559 with optimal sensitivity relative to prior art methods, and can uniquely be used for specifically detecting NIK phosphorylated at T559 and or a target antigen of the present invention.

The antibody can be used for essentially any application benefiting from a reagent capable of binding a target antigen of the present invention with optimal affinity. Such applications include, for example, affinity purification, and hence identification and characterization, of specific ligands of NIK.

As is described and illustrated in the Examples section which follows, an antibody of the present invention capable of specifically binding a phosphorylated polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3, 4, 5 and 6 can be used for specifically detecting, according the guidance set forth therein, the phosphorylated peptides and proteins amino acid sequence set forth in SEQ ID NO: 3, 4, and 5 and 6 respectively.

Preferably, an antibody of the present invention capable of specifically binding a target antigen of the present invention is derived from sera of animals immunized with such a target antigen (referred to hereinafter as "anti-sera"). As is described and illustrated in the Examples section below, an anti-serum of the present invention capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 3 can

be generated by immunizing a mammal with the target antigen according to the protocol set forth therein. Further guidance for generating the preparation by immunization of a mammal is provided herein below.

5 Guidance for obtaining a polypeptide such as the target antigen is provided herein below.

Depending on the application and purpose, the preparation may be advantageously employed in the form an unpurified anti-serum, or it may be purified in various ways prior to use. For example, the preparation may be used in the form of a  
10 purified: (i) preparation of an antibody of a specific isotype, or set of isotypes; (ii) preparation of an antibody or antibody fragment capable of specifically binding specifically phosphorylated peptides of amino acid sequence set forth in SEQ ID NO: 3, 4, 5 and/or 6 (ii) preparation of an antibody or antibody fragment capable of binding with a desired affinity a target antigen of the present invention.

15 A preparation of the present invention in the form of an unpurified anti-serum can be convenient and satisfactory for use in various applications. For example, as is described and illustrated in the Examples section below, an unpurified anti-serum of the present invention, in particular an unpurified anti-serum generated by immunization with the amino acid sequence set forth in SEQ ID NO: 3 can be used for  
20 efficiently detecting phosphorylated NIK via ELISA analysis. In general, for applications benefiting from a preparation of the present invention capable of binding a target antigen of the present invention with a range of affinities/specificities is desirable, an unpurified antibody of the present invention, such as an anti-serum of the present invention, will be advantageous. Such an unpurified preparation may often be  
25 adequate for a given application since the heterogeneity of the polyclonal antibody or antibody fragment mixture contained therein will often include one or more antibodies or antibody fragments having an adequate binding affinity/specificity for the target antigen.

Alternately, a purified antibody or antibody fragment of the present invention  
30 may be advantageously employed in applications such as those involving administration of the antibody or antibody fragment to an individual, and in those in which detection of the target antigen with optimal sensitivity is desirable.

As used herein, the term "individual", refers to a human.

For example, an IgG antibody preparation of the present invention may be advantageously purified from an anti-serum of the present invention using protein-G affinity purification, preferably via protein-G immunoprecipitation. As is described and illustrated in the Examples section which follows, an anti-serum of the present invention derived from an animal immunized with the phosphorylated peptide of amino acid sequence set forth in SEQ ID NO: 3 and purified via protein-G immunoprecipitation according to the protocol set forth therein, can be used for detecting with optimal sensitivity, via Western immunoblotting analysis, Immunoprecipitation and ELISA the phosphorylated polypeptides with amino acid sequence set forth in SEQ ID NO: 3, 4, 5 and 6.

A purified antibody or antibody fragment of the present invention capable of specifically binding the target antigen can be advantageously used for regulating with optimal specificity a biochemical activity of NIK associated with the target antigen, and for detecting the target antigen with optimal specificity. In particular, a purified antibody or antibody fragment of the present invention capable of specifically binding a target antigen of the present invention comprised within the amino acid sequence set forth in SEQ ID NO: 3, 4, 5 and 6 can be advantageously used for regulating kinase activity of NIK with optimal specificity. In general, for applications benefiting from optimal reproducibility, standardization, or precision, a purified antibody or antibody fragment of the present invention capable of specifically binding the target antigen will generally be optimal relative to an unpurified preparation of the present invention.

Purifying the antibody or antibody fragment capable of specifically binding the target antigen can be achieved, for example, by purifying a preparation of the present invention, such as an unpurified anti-serum of the present invention, via affinity chromatography using a substrate covalently attached to the target antigen. Such a substrate-attached target antigen can be used, according to standard affinity chromatography methodology, for selectively capturing the antibody or antibody fragment capable of specifically binding the target antigen.

The substrate is preferably an affinity chromatography matrix. An affinity chromatography matrix, being a substrate optimized for performing affinity chromatography, may be advantageously employed for achieving optimal affinity

purification.

Substrates having various structural and chemical characteristics may be employed for performing the purification.

Preferably, the substrate comprises a carbohydrate or a derivative thereof.

5 Preferably, the carbohydrate is agarose, sepharose, or cellulose.

Preferably, the substrate is a bead, a resin, or a plastic surface.

Substrates such as beads, resins, or plastic surfaces comprising carbohydrates such as agarose, sepharose or cellulose are routinely used for practicing affinity chromatography in the art.

10 Ample guidance for practicing affinity chromatography, such as that employing such substrates, is provided in the literature of the art (for example, refer to: Wilchek M. and Chaiken I., 2000. *Methods Mol Biol.* 147:1-6; Jack GW. Immunoaffinity chromatography. *Mol Biotechnol* 1, 59-86; Narayanan SR., 1994. *Journal of Chromatography A* 658:237-258; Nisnevitch M. and Firer MA., 2001. *J Biochem*  
15 *Biophys Methods* 49:467-80; Janson JC. & Kristiansen T. in: "Packings and Stationary Phases in Chromatography Techniques" (ed. Unger, KK.) pp. 747 (Marcel Dekker, New York, 1990); Clonis, Y. D. in: "HPLC of Macromolecules: A Practical Approach", pp. 157 (IRL Press, Oxford, 1989); Nilsson J. *et al.*, 1997. *Protein Expr Purif.* 11:1-16).

20 Alternately, a preparation of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

25 An antibody or antibody fragment of the present invention capable of specifically binding a target antigen of the present invention with a desired affinity can be advantageously used for achieving a desired level of regulation of a biochemical activity of NIK associated with the target antigen, and can advantageously be used for detecting the target antigen with desired sensitivity. In particular, an antibody or  
30 antibody fragment of the present invention capable of specifically binding with maximal affinity a target antigen of the present invention comprised in the kinase region of NIK can be advantageously used for optimally down-regulating kinase



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Substrates having various structural and chemical characteristics may be employed for performing the purification.

Preferably, the substrate comprises a carbohydrate or a derivative thereof.

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15 *Biophys Methods* 49:467-80; Janson JC. & Kristiansen T. in: "Packings and Stationary Phases in Chromatography Techniques" (ed. Unger, KK.) pp. 747 (Marcel Dekker, New York, 1990); Clonis, Y. D. in: "HPLC of Macromolecules: A Practical Approach", pp. 157 (IRL Press, Oxford, 1989); Nilsson J. *et al.*, 1997. *Protein Expr Purif.* 11:1-16).

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30 antibody fragment of the present invention capable of specifically binding with maximal affinity a target antigen of the present invention comprised in the kinase region of NIK can be advantageously used for optimally down-regulating kinase

activity of NIK. Similarly, an antibody or antibody fragment of the present invention capable of specifically binding the target antigen with maximal affinity can be advantageously used for detecting the target antigen with optimal sensitivity.

Purifying the antibody or antibody fragment capable of binding the target antigen with a desired affinity from a preparation of the present invention, such as an unpurified anti-serum of the present invention, can be achieved, for example, via affinity chromatography purification of an unpurified—or more preferably a protein-G purified—anti-serum of the present invention, by using the target antigen as an affinity ligand, and via selective elution of a substrate-bound antibody or antibody fragment under conditions of controlled stringency (for example under conditions of controlled pH and/or salt concentration). In particular, an antibody or antibody fragment of the present invention capable of binding the target antigen with a maximal affinity may be conveniently obtained by elution under conditions of effectively maximal stringency (for example under conditions of effectively maximal or minimal pH and/or maximal salt concentration). Typically, an antibody or antibody fragment may be bound to a substrate-attached cognate antigen thereof under conditions of physiological pH and salt concentration, and such an antibody or antibody fragment may typically be eluted from the substrate by decreasing the pH to 2.5 or lower, or by increasing the pH to 11 or higher.

It will be appreciated by the ordinarily skilled artisan that an antibody or antibody fragment having an affinity characterized by a dissociation constant of up to  $10^{-12}$  for a cognate antigen can be obtained using common art techniques.

As described hereinabove, the preparation may advantageously comprise an antibody or antibody fragment attached to any of various types of detectable molecule.

A preparation of the present invention comprising an antibody or antibody fragment attached to a detectable molecule can be used for detecting the target antigen specifically bound by the antibody or antibody fragment.

The preparation may comprise an antibody or antibody fragment attached to any of numerous types of detectable molecule, depending on the application and purpose.

For example, depending on the application and purpose, the detectable molecule may advantageously be a fluorophore, an enzyme, a light-emitting molecule,

or a radioisotope.

Preferably, the detectable molecule is an enzyme.

An enzyme may be advantageously utilized for enabling detection of the target antigen via any of various enzyme-based detection methods. Examples of such methods include, but are not limited to, enzyme linked immunosorbent assay (ELISA; for example, for detecting the target antigen in a solution), enzyme-linked chemiluminescence assay (for example, for detecting the complex in an electrophoretically separated protein mixture), and enzyme-linked histochemical assay (for example, for detecting the complex in a fixed tissue).

As is described and illustrated in the Examples section below, a preparation of the present invention comprising an antibody attached to an enzyme can be used for efficiently detecting NIK via Western immunoblotting analysis or ELISA.

Numerous types of enzymes may be employed for detecting the target antigen, depending on the application and purpose.

Examples of suitable enzymes include, but are not limited to, horseradish peroxidase (HPR),  $\beta$ -galactosidase, and alkaline phosphatase (AP).

Ample guidance for practicing enzyme-based molecular detection methods is provided in the literature of the art (for example, refer to: Khatkhatay MI. and Desai M., 1999. J Immunoassay 20:151-83; Wisdom GB., 1994. Methods Mol Biol. 32:433-40; Ishikawa E. *et al.*, 1983. J Immunoassay 4:209-327; Oellerich M., 1980. J Clin Chem Clin Biochem. 18:197-208; Schuurs AH. and van Weemen BK., 1980. J Immunoassay 1:229-49).

A preparation of the present invention comprising an antibody or antibody fragment attached to a fluorophore may be advantageously employed for detecting the target antigen via any of numerous fluorescence-based molecular detection methods. Depending on the application and purpose, such methods include, but are not limited to, fluorescence activated flow cytometry (FACS; for example for characterizing expression or display of the target antigen in a suspended cell population), fluorescence confocal microscopy (for example, for detecting the molecule in a dead or living cell or tissue in three dimensions), fluorescence *in-situ* hybridization (FISH), fluorescence resonance energy transfer (FRET; for example, for detecting a specific intermolecular association involving the target antigen), fluorescence histochemistry (for example, for

detecting the molecule in a fixed histological sample), and the like.

Various types of fluorophores, depending on the application and purpose, may be employed for detecting the target antigen.

5 Examples of suitable fluorophores include, but are not limited to, phycoerythrin, fluorescein isothiocyanate (FITC), Cy-chrome, rhodamine, green fluorescent protein (GFP), blue fluorescent protein (BFP), Texas red, and the like.

Ample guidance regarding fluorophore selection, methods of linking fluorophores to various types of molecules, such as an antibody or antibody fragment of the present invention, and methods of using such fluorescent immunoconjugates for  
10 detecting molecules is available in the literature of the art [for example, refer to: Richard P. Haugland, "Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals 1992-1994", 5th ed., Molecular Probes, Inc. (1994); U.S. Pat. No. 6,037,137 to Oncoimmunin Inc.; Hermanson, "Bioconjugate Techniques", Academic Press New York, N.Y. (1995); Kay M. *et al.*, 1995. *Biochemistry* 34:293; Stubbs *et al.*,  
15 1996. *Biochemistry* 35:937; Gakamsky D. *et al.*, "Evaluating Receptor Stoichiometry by Fluorescence Resonance Energy Transfer," in "Receptors: A Practical Approach," 2nd ed., Stanford C. and Horton R. (eds.), Oxford University Press, UK. (2001); U.S. Pat. No. 6,350,466 to Targesome, Inc.].

Examples of suitable light-emitting molecules include luminol.

20 Examples of suitable radioisotopes include [125]iodine, [35]sulfur, [3]hydrogen, [32]phosphorus, etc.

The detectable molecule may be attached to the antibody or antibody fragment in various ways, depending on the application and purpose, and on the nature of the molecules involved. Ample guidance for attaching a detectable molecule to an  
25 antibody or antibody fragment is provided in the literature of the art [for example, refer to: "Using Antibodies: A Laboratory Manual", Ed Harlow, David Lane (eds.), Cold Spring Harbor Laboratory Press (1999); also, refer to the extensive guidelines provided by The American Chemical Society, for example at: <http://www.chemistry.org/portal/Chemistry>]. One of ordinary skill in the art, such as a  
30 chemist, will possess the required expertise for suitably practicing such chemical synthesis techniques.

Accordingly, a preparation of the present invention comprising an antibody or

antibody fragment attached to a detectable molecule can be used for efficiently and uniquely detecting the target antigen in essentially any context.

Depending on the application and purpose, the preparation may advantageously be a preparation of any of various types of antibody fragments.

5 The antibody fragment is preferably a single-chain Fv (scFv), or more preferably an Fab, Fab', F(ab')<sub>2</sub> or CDR.

10 An antibody fragment has the advantage of being smaller than a parental antibody from which it is derived while retaining substantially identical target-antigen binding specificity, or both binding specificity and binding affinity, as the parental antibody. Thus, an antibody fragment, by virtue of being smaller than the parental antibody, will thereby generally have superior biodistribution, and diffusion properties (for example, systemically *in-vivo*, or in isolated tissues) than the latter. An antibody fragment substantially lacking an Fc region, such as a single-chain Fv, an Fab', an Fab an F(ab')<sub>2</sub> or a CDR, is advantageous for applications involving exposure of the  
15 preparation to a molecule capable of specifically binding such an Fc region, and in which such binding is undesirable. Typically this may involve an undesired binding of an Fc region exposed to a cognate Fc receptor, or an Fc-binding complement component (for example, complement component C1q, present in serum). Fc receptors are displayed on the surface of numerous immune cell types, including: professional  
20 APCs, such as dendritic cells; B lymphocytes; and granulocytes such as neutrophils, basophils, eosinophils, monocytes, macrophages, and mast cells. Thus, the absence of an Fc region from the antibody fragment may be particularly advantageous for avoiding undesired an Fc receptor-mediated immune cell activation or a complement component-mediated complement cascade, particularly when administering the  
25 preparation *in-vivo* to an individual.

An F(ab')<sub>2</sub> is a fragment of an antibody molecule containing a divalent antigen-binding portion of an antibody molecule.

An F(ab')<sub>2</sub> preparation of the present invention may be conveniently obtained using standard art methods by treating an antibody preparation of the present invention,  
30 such as an anti-serum of the present invention, with the enzyme pepsin. The resultant F(ab')<sub>2</sub> product is a 5S particle.

An Fab, or Fab' is a fragment of an antibody molecule containing a monovalent

antigen-binding portion of an antibody.

The CDR can be generated e.g. as described in EP0585939 or as described by Strandberg et al. (Protein Eng. 2001 Jan; 14(1): 67-74). The CDR according to the invention can be a modified CDR, which has enhanced effect on the modulation of NIK. An example for methods of modification of active peptides is described by Sawa et al. 1999 (J. Med. Chem. 42, 3289-3299).

An Fab' preparation of the present invention may be conveniently obtained using standard art methods by treating an antibody preparation of the present invention, such as an anti-serum of the present invention, with the enzyme pepsin, followed by reduction of the resultant  $F(ab')_2$  into. Such reduction may be effected using a thiol reducing agent, and optionally using a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages. Such treatment generates two monovalent 3.5S Fab's an Fc fragment.

An Fab preparation may be conveniently obtained using standard art methods by treating an antibody preparation of the present invention, such as an anti-serum of the present invention, with the enzyme papain to yield the intact light chain and a portion of heavy chain composed of the variable and  $C_H1$  domains.

Ample guidance for generating an antibody fragment by enzymatic treatment of an antibody is provided in the literature of the art (for example, refer to: Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647; Porter RR., 1959. Biochem J. 73:119-126).

A single chain Fv (also referred to in the art as "scFv") is a single chain molecule including the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker.

An  $F(ab')_2$ , Fab', Fab, or single-chain Fv or CDR preparation of the present invention may be obtained using recombinant techniques.

Preferably, obtaining a recombinant antibody fragment is effected by isolating mRNA of B lymphocytes of animals immunized with the target antigen, generating cDNA from the mRNA via RT-PCR, and using the cDNA to construct an antibody fragment phage-display library. B lymphocytes can be conveniently isolated from the spleen, or, alternately from the blood, bone-marrow, or lymph nodes of the immunized

animal.

Recombinant phages displaying an antibody fragment possessing a desirable target-antigen-binding property can be selected from the library by sequential enrichment of phages having such a binding property from a large excess of non-binding clones. This selection can be achieved using any of various techniques including panning on immobilized target antigen; panning using specific elution; using biotinylated antigen; affinity purification on columns; or direct panning on cells. Following selection, the phages displaying non-specific antibody fragments may be removed by washing and the bound phages, bearing scFv displaying the desired target-antigen binding property, are eluted and amplified by infection of *E. coli*. Once a recombinant phage displaying an antibody fragment having a desired target antigen-binding property has been isolated, the polynucleotide sequence encoding the variable regions of the antibody fragment can be recovered from the phage display package and cloned into a recombinant prokaryotic or eukaryotic expression vector using standard methodology [for example, refer to: "Current Protocols in Molecular Cloning", Ausubel *et al.* (eds.), Greene Publishing and Wiley Interscience, New York, N.Y. (1989); Sambrook *et al.*, *infra* and associated references]. Such a prokaryotic expression vector can be used for producing the purified recombinant antibody fragment in *E. coli* (for example, refer to Studier *et al.*, 1990. *Methods in Enzymol.* 185:60-89). Such a eukaryotic expression vector can be used for genetically transforming a eukaryotic cell for expression of the recombinant antibody fragment.

Ample guidance for obtaining and exploiting an antibody fragment-phage display library from B lymphocyte mRNA is provided in the literature of the art [for example, refer to: Hoogenboom *et al.*, 1998. *Immunotechnology* 4:1-20; Kand *et al.*, 1991. *Proc Natl Acad Sci U S A.* 88:4363; Barbas *et al.* 1991. *Proc Natl Acad Sci U S A.* 88:7978; Garrard *et al.*, 1991. *Biotechnology* 9:1373-1377; Hoogenboom *et al.*, 1991. *Nucleic Acids Res.* 19:4133-4137; Sharon *et al.*, 2000. *Combinational Chemistry and High Throughput Screening* 3:185-196; U.S. Pat. Nos. 5,698,426, 5,658,727, 5,223,409, 5,403,484, 5,580,717, 5,427,908, 5,750,753, 5,821,047, 5,571,698, 5,427,908, 5,516,637, 5,780,225, 5,658,727, 5,733,743 and 5,969,108; PCT application No. PCT/GB91/01134; PCT publications WO 90/02809, WO 91/10737, WO 92/01047, WO 92/18619, WO 93/11236, WO 95/15982, and WO 95/20401; Brinkman

*et al.*, 1995. J. Immunol. Methods 182:41-50; Ames *et al.*, 1995. J. Immunol. Methods 184:177-186; Kettleborough *et al.*, 1994. Eur. J. Immunol. 24:952-958; Persic *et al.*, 1997. Gene 187 9-18; and Burton *et al.*, 1994. Advances in Immunology 57:191-280; Pluckthun in: "The Pharmacology of Monoclonal Antibodies", Vol. 113, Rosenberg and Moors (eds.), Springer-Verlag, New York, pp. 269-315 (1994); Hoogenboom *et al.*, 1998. Immunotechnology 4:1-20].

It will be appreciated that the above-described methodology can be used to obtain a monoclonal antibody fragment preparation of the present invention having essentially any desired target antigen-binding affinity and/or specificity. Such a preparation can be utilized in various applications benefiting from a reagent capable of binding the target antigen with such defined target antigen-binding characteristics.

The present invention provides a method for preparing a monoclonal antibody comprising growing a cloned hybridoma comprising a spleen cell from a mammal immunized with an amino acid sequence comprising SEQ ID NO: 6, or a portion thereof e.g. that set forth in SEQ ID NO: 3 wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 and a homogeneous or heterogeneous lymphoid cell in liquid medium or mammalian abdomen to allow the hybridoma to produce and accumulate the monoclonal antibody.

An example of hybridoma to be used for the preparation of the monoclonal antibodies is the hybridoma clone Nik-P4 30.12 deposited at the CNCM under No. I-3095.

Since an Fab' is essentially similar in structure to an Fab, a preparation of the present invention comprising an Fab' may be employed essentially interchangeably with one comprising an Fab, where such Fab' and Fab comprise essentially the same heavy and light chain variable regions. For applications, as will usually be the case, benefiting from a preparation of the present invention comprising an antibody fragment capable of binding the target antigen with maximal affinity, an F(ab')<sub>2</sub> preparation of the present invention may superior to an Fab, Fab' or scFv preparation of the present invention, due to the divalent binding of an F(ab')<sub>2</sub> to the target antigen relative to the monovalent binding of such a monovalent antibody fragment.



As mentioned hereinabove, depending on the application and purpose, the antibody or antibody fragment preparation may originate from any of various mammalian species

5 An antibody or antibody fragment preparation of the present invention originating from a desired species may be derived from serum of the animal of such species immunized with the target antigen.

Preferably, the antibody or antibody fragment is of mouse origin.

10 A preparation of the present invention of a human or humanized antibody or antibody fragment may be preferable for applications involving administration of the preparation to an individual. For example, a human or humanized antibody or antibody fragment will generally tend to be optimally tolerated immunologically, and hence will display an optimal half-life *in-vivo* in a human, and will thereby display optimal effectiveness. Further guidance regarding production and exploitation of human or humanized antibodies is provided hereinbelow.

15 The preparation may be used *per se* or it can be formulated as an active ingredient in a pharmaceutical composition.

Thus, according to the present invention there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active ingredient, the antibody or antibody fragment of the present invention.

20 Methods of formulating the antibody or antibody fragment of the present invention as an active ingredient in a pharmaceutical composition, and methods of exploiting such a pharmaceutical composition are described hereinbelow.

25 As described hereinabove, by virtue of its capacity to specifically bind a region, such as a functional region, of NIK, the antibody or antibody fragment of the present invention can be used for regulating a biochemical activity of a NIK molecule associated with such a functional region.

30 Thus, according to yet another aspect of the present invention there is provided a method of regulating a biochemical activity of a NIK molecule. The method is effected by contacting the NIK molecule with an antibody or antibody fragment of the present invention.

Preferably, the method is used for regulating the biochemical activity in a human NIK molecule.

As described hereinabove, since the antibody or antibody fragment of the present invention is capable of specifically binding phosphorylated NIK in such a way as to down-regulate NIK kinase activity, and since such activity is required for activation of NF- $\kappa$ B, as extensively elaborated in the "Field and Background of the Invention" section above, the method can be used for optimally down-regulating NF- $\kappa$ B activity.

Hence, by virtue of enabling optimal down-regulation of NF- $\kappa$ B activity, the method can be used for optimally treating in an individual a disease associated with deregulated NF- $\kappa$ B activity, in particular excessive or constitutive NF- $\kappa$ B activity.

When using the method according to this aspect of the present invention for treating the disease in the individual, contacting the NIK molecule with the antibody or antibody fragment may be advantageously effected by administering the antibody or antibody fragment to an individual.

Preferably, administering the antibody or antibody fragment is effected by administering the pharmaceutical composition of the present invention comprising the antibody or antibody fragment of the present invention as an active ingredient.

The antibody or antibody fragment is preferably administered so as to achieve a sufficient level of antibody fragment bound to the target antigen so as to achieve a desired regulation of the biochemical activity.

An ordinarily skilled artisan, such as a physician, more preferably a physician specialized in the disease, will possess the required expertise for determining a suitable therapeutic protocol, including a suitable route of administration, and a suitable dosage of the antibody or antibody fragment for effectively treating the disease according to the teachings of the present invention.

NIK is normally an intracellular molecule, and, as such, in order to optimally contact the antibody or antibody fragment with the NIK molecule in a cell, such as a cell characterized by deregulated NF- $\kappa$ B activity, the method is preferably practiced in such a way as to facilitate contact of the antibody or antibody fragment with the NIK molecule within the cell.

Such intracellular contacting may be facilitated in various ways, depending on the application and purpose.

For example, such intracellular contacting may be effected by contacting the

cell with the antibody or antibody fragment in association with a lipid-based carrier capable of facilitating penetration of the antibody or antibody fragment inside the cell.

Alternatively, such intracellular contacting may be effected by genetically transforming the cell with an expression vector capable of expressing the antibody  
5 fragment of the present invention intracellularly.

Suitable types of lipid carriers for facilitating entry of the antibody or antibody fragment into the cell include liposomes, and immunoliposomes. Immunoliposomes, by virtue of enabling cell-type specific delivery of molecules may be advantageously employed for selectively delivering the antibody or antibody fragment in cells affected  
10 by disease, where such cells express distinctive surface antigens, such as markers of inflammation in cells displaying a pathological immune response (for example, CD25 or CD69 in activated T lymphocytes), or tumor-associated antigens in malignant cells (for example, HER-2 in adenocarcinoma cells, MAGE-1 in melanoma cells, and the like).

Ample guidance for using such lipid-based carriers for intracellular delivery of  
15 therapeutic molecules, such as an antibody or antibody fragment of the present invention, to diseased cells is provided in the literature of the art (for example, refer to: Abra RM. *et al.*, 2002. *J Liposome Res.* 12:1-3; Park JW., 2002. *Breast Cancer Res.*;4(3):95-9; Bendas G., 2001. *BioDrugs* 15:215-24; Maruyama K., 2000. *Biol Pharm Bull.* 23:791-9; Hong K. *et al.*, 1999. *Ann N Y Acad Sci.* 886:293-6; Margalit R., 1995. *Crit Rev Ther Drug Carrier Syst.* 12:233-61; Storm G. and Crommelin DJ.,  
20 1997. *Hybridoma* 16:119-25; Park JW. *et al.*, 1997. *Adv Pharmacol.* 40:399-435).

Producing a recombinant antibody fragment, such as the recombinant antibody fragment of the present invention, intracellularly is routinely practiced in the  
25 art. A recombinant antibody fragment expressed intracellularly may be referred to as an "intrabody" in the art. Ample guidance for using intracellular expression of a recombinant antibody fragment capable of specifically binding a biomolecule for regulating a biochemical activity, such as an enzymatic activity, of the biomolecule in a cell is provided in the literature of the art (for example, refer to: Mhashilkar AM. *et al.*, 2002. *Gene Ther.* 9:307-19; Arafat W. *et al.*, 2000. *Cancer Gene Ther.* 7:1250-6; Cohen PA. *et al.*, 1998. *Oncogene* 17:2445-56; Hassanzadeh Gh G. *et al.*, 1998. *FEBS Lett.* 437:81-6; Richardson JH. *et al.*, 1998. *Gene Ther.* 5:635-44; for general guidance  
30

for expressing a recombinant antibody fragment in a cell, refer, for example, to: der Maur AA. *et al.*, 2002. J Biol Chem. 277:45075-85; Zhu Q. *et al.*, 1999. J Immunol Methods. 231:207-22; Wirtz P. and Steipe B., 1999. Protein Sci. 8:2245-50; Ohage E. and Steipe B., 1999. J Mol Biol. 291:1119-28).

5 Genetically transforming a mammalian cell with an expression vector may be effected using any of various commonly practiced art methods, such as stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Ample guidance for practicing such methods is provided in the literature of the art [for example, refer to: Sambrook *et al.*, *infra* and associated references; Chang  
10 *et al.* in: "Somatic Gene Therapy", CRC Press, Ann Arbor, MI (1995); Vega *et al.* in: "Gene Targeting", CRC Press, Ann Arbor MI. (1995); Vectors, A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston MA (1988); Gilboa *et al.*, 1986. Biotechniques 4:504-512; for vectors involving the central nervous system refer, for example, to United States patent 4,866,042; for positive-negative selection methods for  
15 inducing homologous recombination, refer, for example, to: United States patents 5,464,764 and 5,487,992].

Further guidance regarding production and exploitation of expression vectors for expressing an antibody fragment of the present invention in a cell is provided hereinbelow.

20 As described hereinabove, the method according to this aspect of the present invention can be used for optimally treating a disease associated with deregulated NF- $\kappa$ B activity. NF- $\kappa$ B activity is generally involved in activation of a very broad range of immune responses, including those triggered by: lymphocyte antigen receptors, lymphocyte costimulatory receptors, TNF receptors, interleukin receptors,  
25 LMP1, RANK, human Toll receptor, and lipopolysaccharide (LPS). Since such receptors are involved in mediating a very broad range of types of immune responses, the method according to this aspect of the present invention can be used for treating numerous diseases whose pathogenesis is associated with such immune responses. Such diseases include autoimmune diseases, inflammatory diseases, transplantation-  
30 related diseases, and allergic diseases. For example NF- $\kappa$ B has been shown to be involved in the pathogenesis of diverse examples of such diseases including allergic diseases such as asthma, autoimmune diseases such as rheumatoid arthritis,

inflammatory disease such as inflammatory bowel disease, atherosclerosis, and Alzheimer's disease, and transplantation-related diseases such as graft rejection. Furthermore, deregulated NF- $\kappa$ B signaling has been shown to be associated with various malignant diseases.

5 As such, the method according to this aspect of the present invention can be used for effectively treating diseases such as autoimmune, inflammatory, transplantation-related, allergic, and malignant diseases.

Specific examples of diseases which can be treated according to this aspect of the present invention are listed hereinbelow.

10 As described hereinabove, the target antigen, which is a polypeptide, may be obtained in various ways.

Preferably, the target antigen is obtained via standard chemical synthesis methodology.

15 Alternatively, the target antigen may be obtained by proteolytic cleavage of naturally expressed NIK, or may be obtained via standard recombinant techniques using *in-vitro* expression systems (for example, refer to Sambrook *et al.* *infra*, and associated references).

20 The target antigen may be chemically synthesized using, for example, standard solid phase techniques. Such techniques include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. Solid phase polypeptide synthesis procedures are well known in the art [for example, refer to Stewart *et al.*, in "Solid Phase Peptide Synthesis", 2nd ed., Pierce Chemical Company, (1984)].

25 A synthetic polypeptide can be purified by preparative high performance liquid chromatography procedure, such as described by Creighton T. [Proteins, structures and molecular principles, W. H. Freeman and Co. N.Y. (1983)] and its amino acid sequence may be confirmed via standard amino acid sequencing procedures.

As described hereinabove, the preparation is preferably derived by immunizing a mammal with the target antigen.

30 Generating the preparation *in-vivo* may be advantageously effected by repeated injection of the target antigen into a mammal in the presence of adjuvant according to a schedule which boosts production of antibodies in the serum. In cases wherein the

target antigen is too small to elicit an adequate immunogenic response (referred to as a "hapten" in the art), the hapten can be coupled to an antigenically neutral carrier such as keyhole limpet hemocyanin (KLH) or serum albumin [e.g., bovine serum albumin (BSA)] carriers (for example, refer to US. Pat. Nos. 5,189,178 and 5,239,078).

5 Coupling a hapten to a carrier can be effected using various methods well known in the art. For example, direct coupling to amino groups can be effected and optionally followed by reduction of the imino linkage formed. Alternatively, the carrier can be coupled using condensing agents such as dicyclohexyl carbodiimide or other carbodiimide dehydrating agents. Linker compounds can also be used to effect the  
10 coupling; both homobifunctional and heterobifunctional linkers are available from Pierce Chemical Company, Rockford, Ill. The resulting immunogenic complex can then be injected into suitable mammalian subjects such as mice, rabbits, and the like. Following *in-vivo* generation of an antibody, its serum titer in the host mammal can readily be measured using immunoassay procedures which are well known in the art.

15 As described hereinabove, the preparation may advantageously comprise a humanized antibody or antibody fragment.

Humanized antibodies or antibody fragments are genetically engineered chimeric antibodies or antibody fragments having—preferably minimal—portions derived from non human antibodies. Humanized antibodies include antibodies in  
20 which complementary determining regions of a human antibody (recipient antibody) are replaced by residues from a complementarity determining region of a non human species (donor antibody) such as mouse, rat or rabbit having the desired functionality. In some instances, Fv framework residues of the human antibody are replaced by corresponding non human residues. Humanized antibodies may also comprise residues  
25 which are found neither in the recipient antibody nor in the imported complementarity determining region or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the complementarity determining regions correspond to those of a non-human antibody and all, or substantially all, of the framework regions  
30 correspond to those of a relevant human consensus sequence. Humanized antibodies optimally also include at least a portion of an antibody constant region, such as an Fc region, typically derived from a human antibody (see, for example, Jones *et al.*, 1986.

Nature 321:522-525; Riechmann *et al.*, 1988. Nature 332:323-329; and Presta, 1992. Curr. Op. Struct. Biol. 2:593-596). Methods for humanizing non human antibodies or antibody fragments are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non human.

5 These non human amino acid residues are often referred to as imported residues which are typically taken from an imported variable domain. Humanization can be essentially performed as described (see, for example: Jones *et al.*, 1986. Nature 321:522-525; Riechmann *et al.*, 1988. Nature 332:323-327; Verhoeven *et al.*, 1988. Science 239:1534-1536; U.S. Pat. No. 4,816,567) by substituting human  
10 complementarity determining regions with corresponding rodent complementarity determining regions. Accordingly, such humanized antibodies are chimeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non human species. In practice, humanized antibodies may be typically human antibodies in which some complementarity  
15 determining region residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies. Human antibodies or antibody fragments can also be produced using various techniques known in the art, including phage display libraries [see, for example, Hoogenboom and Winter, 1991. J. Mol. Biol. 227:381; Marks *et al.*, 1991. J. Mol. Biol. 222:581; Cole *et al.*, "Monoclonal  
20 Antibodies and Cancer Therapy", Alan R. Liss, pp. 77 (1985); Boerner *et al.*, 1991. J. Immunol. 147:86-95). Humanized antibodies can also be made by introducing sequences encoding human immunoglobulin loci into transgenic animals, e.g., into mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon antigenic challenge, human antibody production is  
25 observed in such animals which closely resembles that seen in humans in all respects, including gene rearrangement, chain assembly, and antibody repertoire. Ample guidance for practicing such an approach is provided in the literature of the art (for example, refer to: U.S. Pat. Nos. 5,545,807, 5,545,806, 5,569,825, 5,625,126, 5,633,425, and 5,661,016; Marks *et al.*, 1992. Bio/Technology 10:779-783; Lonberg *et al.*,  
30 *et al.*, 1994. Nature 368:856-859; Morrison, 1994. Nature 368:812-13; Fishwild *et al.*, 1996. Nature Biotechnology 14:845-51; Neuberger, 1996. Nature Biotechnology 14:826; Lonberg and Huszar, 1995. Intern. Rev. Immunol. 13:65-93).

As described hereinabove, an antibody fragment of the present invention may be advantageously expressed intracellularly using an expression vector.

A preferred approach for genetically modifying a cell of an individual with an expression vector is using a viral vector. Viral vectors offer several advantages including higher efficiency of transformation, and targeting to, and propagation in, specific cell types. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through specific cell receptors, such as cancer cell receptors. Such targeting capacity can be used for directing expression of an antibody or antibody fragment of the present invention in a cell characterized by deregulated NF- $\kappa$ B activity.

Retroviral vectors represent one class of vectors suitable for use with the present invention. Defective retroviruses are routinely used as vectors for genetically modifying mammalian cells, such as epithelial cells endothelial cells, lymphocytes, myoblasts, hepatocytes and bone marrow cells, to express recombinant proteins. Portions of the retroviral genome can be removed to render the retrovirus replication defective and the replication defective retrovirus can then packaged into virions, which can be used to infect target cells through the use of a helper virus and while employing standard techniques. Ample guidance for generating a retroviral vector capable of expressing a recombinant protein in a mammalian cell is provided in the literature of the art (for example, refer to Miller, A.D., 1990. *Blood* 76: 271; Sambrook *et al.*, *infra* and associated references).

Another suitable expression vector may be an adenoviral vector. Adenoviral vectors are extensively studied and routinely used gene transfer vectors. Key advantages of adenoviral vectors include relatively high transduction efficiency of dividing and quiescent cells, natural tropism to a wide range of epithelial tissues and facile production of high titers. The adenovirus DNA is transported to the nucleus, but does not integrate thereinto. Thus the risk of mutagenesis with adenoviral vectors is minimized. Ample guidance for producing and exploiting adenoviral vectors to treat diseases is provided in the literature of the art [for example, refer to: Russel, W.C., 2000. *J. Gen. Virol.* 81:57-63; for guidance regarding use of adenoviral vectors cancer treatment, refer, for example, to Seth *et al.*, Adenoviral vectors for cancer gene therapy. In: P. Seth (ed.) *Adenoviruses: Basic biology to Gene Therapy*, Landes, Austin, TX, pp. 103-120 (1999)].



A specific example of a suitable adenoviral vector is the adenovirus-derived vector Ad-TK. This vector expresses a herpesvirus thymidine kinase (TK) gene for either positive or negative selection and includes an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor which includes most cancers of epithelial origin (Sandmair *et al.*, 2000. Hum. Gene. Ther. 11:2197-2205).

A suitable viral expression vector may also be a chimeric adenovirus/retrovirus vector which combines retroviral and adenoviral components, and which has been shown to be more efficient than traditional expression vectors. Ample guidance for producing and exploiting such vectors is provided in the literature of the art (for example, refer to Pan *et al.*, 2002. Cancer Letters 184:179-188).

The expression vector can be administered in various ways. If viral vectors are used the procedure can take advantage of their target specificity and consequently, such vectors may not have to be administered locally at an anatomic site affected by the disease. However, local administration can provide a quicker and more effective treatment. Administration of viral vectors can also be performed by, for example, intravenous or subcutaneous injection into the individual. Following injection, the viral vectors will circulate until they recognize host cells with appropriate target specificity for infection.

As described hereinabove, the present invention provides a pharmaceutical composition comprising the antibody or antibody fragment of the present invention as an active ingredient.

As used herein, the phrase "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of active ingredients to an organism.

Herein the term "active ingredients" refers to the antibody or antibody fragment of the present invention accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not

abrogate the biological activity and properties of the administered active ingredients. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration of the pharmaceutical composition may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injection as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injection.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of the individual.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active ingredients with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active ingredient doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-  
5 tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the active ingredients and a suitable powder base such as lactose or starch.

10 The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain  
15 formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame  
20 oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly  
25 concentrated solutions.

Alternatively, the active ingredients may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using,  
30 e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an

amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (antibody or antibody fragment of the present invention) capable of preventing, alleviating or ameliorating symptoms of the disease, or prolong the survival of the individual being treated.

5 Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in-vitro* and cell culture  
10 assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in-vitro*, in cell cultures or  
15 experimental animals. The data obtained from these *in-vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (for example,  
20 refer to Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma or brain levels of the active ingredients sufficient to exert a desired therapeutic effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from *in-vitro* data. Dosages necessary to achieve the MEC will  
25 depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the  
30 disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the individual being treated, the severity of the affliction, the manner of

administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredients. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising an antibody or antibody fragment of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as if further detailed above.

As described hereinabove, the present invention can be used to treat a disease associated with a pathological immune response.

Examples of such diseases include diseases associated with Type I (immediate or IgE-mediated) hypersensitivity, diseases associated with Type II (antibody-mediated) hypersensitivity, diseases associated with Type IV (T lymphocyte-mediated) hypersensitivity, diseases associated with delayed type hypersensitivity (DTH), autoimmune diseases, and diseases associated with transplantation of a graft.

Further examples of diseases associated with hypersensitivity include, for example, diseases associated with Type III (immune complex-mediated) hypersensitivity, diseases associated with inflammation, diseases associated with infection and diseases associated with idiopathic hypersensitivity.

Examples of Type I hypersensitivity include, but are not limited to, allergic diseases such as asthma, hives, urticaria, pollen allergy, dust mite allergy, venom allergy, cosmetics allergy, latex allergy, chemical allergy, drug allergy, insect bite allergy, animal dander allergy, stinging plant allergy, poison ivy allergy and food allergy.

Examples of Type II hypersensitivity include, but are not limited to, rheumatoid

diseases, rheumatoid autoimmune diseases, rheumatoid arthritis (Krenn V. *et al.*, 2000. *Histol Histopathol.* 15:791), spondylitis, ankylosing spondylitis (Jan Voswinkel *et al.*, 2001. *Arthritis Res.* 3:189), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Erikson J. *et al.*, 1998. *Immunol Res.* 17:49), sclerosis, systemic sclerosis (Renaudineau Y., *et al.*, 1999. *Clin Diagn Lab Immunol.* 6:156);

5 Chan OT. *et al.*, 1999. *Immunol Rev.* 169:107), glandular diseases, glandular autoimmune diseases, pancreatic autoimmune diseases, diabetes, Type I diabetes (Zimmet P. 1996. *Diabetes Res Clin Pract.* 34 Suppl:S125), thyroid diseases, autoimmune thyroid diseases, Graves' disease (Orgiazzi J. 2000. *Endocrinol Metab*

10 *Clin North Am.* 29:339), thyroiditis, spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S. 2000. *J Immunol.* 165(12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.*, *Nippon Rinsho* 1999. 57(8):1810), myxedema, idiopathic myxedema (Mitsuma T. *Nippon Rinsho.* 1999. 57(8):1759); autoimmune reproductive diseases, ovarian diseases, ovarian autoimmunity (Garza KM. *et al.* *J Reprod Immunol.* 1998.

15 37(2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, *Am J Reprod Immunol.* 2000. 43(3):134), repeated fetal loss (Tincani A. *et al.*, *Lupus* 1998. 7 Suppl 2:S107-9), neurodegenerative diseases, neurological diseases, neurological autoimmune diseases, multiple sclerosis (Cross AH. *et al.*, *J Neuroimmunol.* 2001. 112(1-2):1), Alzheimer's disease (Oron L. *et al.*, *J Neural Transm Suppl.* 1997. 49:77),

20 myasthenia gravis (Infante AJ. and Kraig E. *Int Rev Immunol.* 1999. 18(1-2):83), motor neuropathies (Kornberg AJ. *J Clin Neurosci.* 2000. 7(3):191), Guillain-Barre syndrome, neuropathies and autoimmune neuropathies (Kusunoki S. *Am J Med Sci.* 2000. 319(4):234), myasthenic diseases, Lambert-Eaton myasthenic syndrome (Takamori M. *Am J Med Sci.* 2000. 319(4):204), paraneoplastic neurological diseases,

25 cerebellar atrophy, paraneoplastic cerebellar atrophy, non-paraneoplastic stiff man syndrome, cerebellar atrophies, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette syndrome, polyendocrinopathies, autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. *Rev Neurol (Paris)* 2000. 156(1):23); neuropathies, dysimmune

30 neuropathies (Nobile-Orazio E. *et al.*, *Electroencephalogr Clin Neurophysiol Suppl.* 1999. 50:419); neuromyotonia, acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.*, *Ann N Y Acad Sci.* 1998. 841:482), cardiovascular

diseases, cardiovascular autoimmune diseases, atherosclerosis (Matsuura E. *et al.*, Lupus. 1998. 7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998. 7 Suppl 2:S132), thrombosis (Tincani A. *et al.*, Lupus 1998. 7 Suppl 2:S107-9), granulomatosis, Wegener's granulomatosis, arteritis, Takayasu's arteritis and  
 5 Kawasaki syndrome (Praprotnik S. *et al.*, Wien Klin Wochenschr 2000. 112 (15-16):660); anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.*, Semin Thromb Hemost. 2000. 26(2):157); vasculitises, necrotizing small vessel vasculitises, microscopic polyangiitis, Churg and Strauss syndrome, glomerulonephritis, pauci-immune focal necrotizing glomerulonephritis, crescentic glomerulonephritis (Noel LH.  
 10 Ann Med Interne (Paris). 2000. 151(3):178); antiphospholipid syndrome (Flamholz R. *et al.*, J Clin Apheresis 1999. 14(4):171); heart failure, agonist-like  $\beta$ -adrenoceptor antibodies in heart failure (Wallukat G. *et al.*, Am J Cardiol. 1999. 83(12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999. 14(2):114); hemolytic anemia, autoimmune hemolytic anemia (Efremov DG. *et al.*, Leuk Lymphoma 1998.  
 15 28(3-4):285), gastrointestinal diseases, autoimmune diseases of the gastrointestinal tract, intestinal diseases, chronic inflammatory intestinal disease (Garcia Herola A. *et al.*, Gastroenterol Hepatol. 2000 Jan; 23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000. 138(2):122), autoimmune diseases of the musculature, myositis, autoimmune myositis, Sjogren's syndrome (Feist E. *et al.*, Int Arch Allergy  
 20 Immunol. 2000. 123(1):92); smooth muscle autoimmune disease (Zauli D. *et al.*, Biomed Pharmacother. 1999. 53(5-6):234), hepatic diseases, hepatic autoimmune diseases, autoimmune hepatitis (Manns MP. J Hepatol. 2000. 33(2):326) and primary biliary cirrhosis (Strassburg CP. *et al.*, Eur J Gastroenterol Hepatol. 1999. 11(6):595).

Examples of Type III hypersensitivity include, but are not limited to, diseases  
 25 mediated by immune effector cells such as, for example, neutrophils or macrophages activated by, for example, immune complexes via Fc receptors, such as, for example Fc $\gamma$  receptors.

Examples of Type IV hypersensitivity include, but are not limited to, rheumatoid diseases, rheumatoid arthritis (Tisch R and McDevitt HO. Proc Natl Acad  
 30 Sci U S A 1994. 91(2):437), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Datta SK., Lupus 1998. 7(9):591), glandular diseases, glandular autoimmune diseases, pancreatic diseases, pancreatic autoimmune diseases,



Type 1 diabetes (Castano L. and Eisenbarth GS. *Ann. Rev. Immunol.* 8:647); thyroid diseases, autoimmune thyroid diseases, Graves' disease (Sakata S. *et al.*, *Mol Cell Endocrinol.* 1993. 92(1):77); ovarian diseases (Garza KM. *et al.*, *J Reprod Immunol.* 1998. 37(2):87), prostatitis, autoimmune prostatitis (Alexander RB. *et al.*, *Urology* 1997. 50(6):893), polyglandular syndrome, autoimmune polyglandular syndrome, Type I autoimmune polyglandular syndrome (Hara T. *et al.*, *Blood* 1991. 77 (5):1127), neurological diseases, autoimmune neurological diseases, multiple sclerosis, neuritis, optic neuritis (Soderstrom M. *et al.*, *J Neurol Neurosurg Psychiatry* 1994. 57(5):544), myasthenia gravis (Oshima M. *et al.*, *Eur J Immunol.* 1990. 20(12):2563), stiff-man syndrome (Hiemstra HS. *et al.*, *Proc Natl Acad Sci U S A* 2001. 98 (7):3988), cardiovascular diseases, cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.*, *J Clin Invest.* 1996. 98(8):1709), autoimmune thrombocytopenic purpura (Semple JW. *et al.*, *Blood* 1996. 87(10):4245), anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, *Viral Immunol.* 1998. 11(1):9), hemolytic anemia (Sallah S. *et al.*, *Ann Hematol.* 1997. 74(3):139), hepatic diseases, hepatic autoimmune diseases, hepatitis, chronic active hepatitis (Franco A. *et al.*, *Clin Immunol Immunopathol.* 1990. 54(3):382), biliary cirrhosis, primary biliary cirrhosis (Jones DE. *Clin Sci (Colch)* 1996. 91(5):551), nephric diseases, nephric autoimmune diseases, nephritis, interstitial nephritis (Kelly CJ. *J Am Soc Nephrol.* 1990. 1(2):140), connective tissue diseases, ear diseases, autoimmune connective tissue diseases, autoimmune ear disease (Yoo TJ. *et al.*, *Cell Immunol.* 1994. 157(1):249), disease of the inner ear (Gloddek B. *et al.*, *Ann N Y Acad Sci.* 1997. 830:266), skin diseases, cutaneous diseases, dermal diseases, bullous skin diseases, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of diseases associated with delayed type hypersensitivity include, but are not limited to, contact dermatitis and drug eruption.

Examples of autoimmune diseases include, but are not limited to, cardiovascular diseases, rheumatoid diseases, glandular diseases, gastrointestinal diseases, cutaneous diseases, hepatic diseases, neurological diseases, muscular diseases, nephric diseases, diseases related to reproduction, connective tissue diseases and systemic diseases.

Examples of autoimmune cardiovascular diseases include, but are not limited to

atherosclerosis (Matsuura E. *et al.*, Lupus. 1998 7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998. 7 Suppl 2:S132), thrombosis (Tincani A. *et al.*, Lupus 1998. 7 Suppl 2:S107-9), Wegener's granulomatosis, Takayasu's arteritis, Kawasaki syndrome (Praprotnik S. *et al.*, Wien Klin Wochenschr. 2000. 112(15-  
5 16):660), anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.*, Semin Thromb Hemost. 2000. 26(2):157), necrotizing small vessel vasculitis, microscopic polyangiitis, Churg and Strauss syndrome, pauci-immune focal necrotizing and crescentic glomerulonephritis (Noel LH. Ann Med Interne (Paris). 2000. 151(3):178), antiphospholipid syndrome (Flamholz R. *et al.*, J Clin Apheresis 1999. 14(4):171),  
10 antibody-induced heart failure (Wallukat G. *et al.*, (1999) Am J Cardiol. 83(12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999. 14(2):114; Semple JW. *et al.*, Blood 1996. 87(10):4245), autoimmune hemolytic anemia (Efremov DG. *et al.*, Leuk Lymphoma 1998 28(3-4):285; Sallah S. *et al.*, Ann Hematol. 1997. 74(3):139), cardiac autoimmunity in Chagas' disease (Cunha-Neto E.  
15 *et al.*, J Clin Invest. 1996. 98(8):1709) and anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, Viral Immunol. 1998. 11(1):9).

Examples of autoimmune rheumatoid diseases include, but are not limited to rheumatoid arthritis (Krenn V. *et al.*, (2000) Histol Histopathol. 15(3):791; Tisch R, McDevitt HO. (1994) Proc Natl Acad Sci U S A. 91(2):437) and ankylosing  
20 spondylitis (Jan Voswinkel *et al.*, (2001) Arthritis Res. 3(3):189).

Examples of autoimmune glandular diseases include, but are not limited to, pancreatic disease, Type I diabetes, thyroid disease, Graves' disease, thyroiditis, spontaneous autoimmune thyroiditis, Hashimoto's thyroiditis, idiopathic myxedema, ovarian autoimmunity, autoimmune anti-sperm infertility, autoimmune prostatitis and  
25 Type I autoimmune polyglandular syndrome diseases include, but are not limited to autoimmune diseases of the pancreas, Type 1 diabetes (Castano L. and Eisenbarth GS. 1990. Ann. Rev. Immunol. 8:647; Zimmet P. Diabetes Res Clin Pract. 1996. 34 Suppl:S125), autoimmune thyroid diseases, Graves' disease (Orgiazzi J. Endocrinol Metab Clin North Am. 2000. 29(2):339; Sakata S. *et al.*, Mol Cell Endocrinol. 1993.  
30 92(1):77), spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, J Immunol. 2000. 165(12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.*, Nippon Rinsho 1999. 57(8):1810), idiopathic myxedema (Mitsuma T. Nippon Rinsho. 1999.

57(8):1759), ovarian autoimmunity (Garza KM. *et al.*, J Reprod Immunol. 1998. 37(2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, Am J Reprod Immunol. 2000. 43(3):134), autoimmune prostatitis (Alexander RB. *et al.*, Urology 1997, 50(6):893) and Type I autoimmune polyglandular syndrome (Hara T. *et al.*, Blood. 1991. 77 (5):1127).

Examples of autoimmune gastrointestinal diseases include, but are not limited to, chronic inflammatory intestinal diseases (Garcia Herola A. *et al.*, Gastroenterol Hepatol. 2000. 23(1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000. 138(2):122), colitis, ileitis and Crohn's disease.

10        Examples of autoimmune cutaneous diseases include, but are not limited to, autoimmune bullous skin diseases, such as, but are not limited to, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

15        Examples of autoimmune hepatic diseases include, but are not limited to, hepatitis, autoimmune chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol. 1990. 54(3):382), primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996. 91(5):551; Strassburg CP. *et al.*, Eur J Gastroenterol Hepatol. 1999. 11(6):595) and autoimmune hepatitis (Manns MP. J Hepatol. 2000. 33(2):326).

20        Examples of autoimmune neurological diseases include, but are not limited to, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol. 2001. 112(1-2):1), Alzheimer's disease (Oron L. *et al.*, J Neural Transm Suppl. 1997. 49:77), myasthenia gravis (Infante AJ. and Kraig E, Int Rev Immunol. 1999. 18(1-2):83; Oshima M. *et al.*, Eur J Immunol. 1990. 20:2563), neuropathies, motor neuropathies (Kornberg AJ. J Clin Neurosci. 2000. 7:191); Guillain-Barre syndrome and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000. 319(4):234), myasthenia, Lambert-Eaton  
25        myasthenic syndrome (Takamori M. Am J Med Sci. 2000. 319(4):204); paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy and stiff-man syndrome (Hiemstra HS. *et al.*, Proc Natl Acad Sci U S A 2001. 98(7):3988); non-paraneoplastic stiff man syndrome, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la  
30        Tourette syndrome and autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol. (Paris) 2000. 156(1):23); dysimmune neuropathies (Nobile-Orazio E. *et al.*, Electroencephalogr Clin Neurophysiol Suppl. 1999. 50:419); acquired

neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.*, Ann N Y Acad Sci. 1998. 841:482), neuritis, optic neuritis (Soderstrom M. *et al.*, J Neurol Neurosurg Psychiatry 1994. 57(5):544) and neurodegenerative diseases.

5 Examples of autoimmune muscular diseases include, but are not limited to, myositis, autoimmune myositis and primary Sjogren's syndrome (Feist E. *et al.*, Int Arch Allergy Immunol. 2000. 123(1):92) and smooth muscle autoimmune disease (Zauli D. *et al.*, Biomed Pharmacother. 1999. 53(5-6):234).

10 Examples of autoimmune nephric diseases include, but are not limited to, nephritis and autoimmune interstitial nephritis (Kelly CJ. J Am Soc Nephrol. 1990. 1(2):140).

Examples of autoimmune diseases related to reproduction include, but are not limited to, repeated fetal loss (Tincani A. *et al.*, Lupus 1998. 7 Suppl 2:S107-9).

15 Examples of autoimmune connective tissue diseases include, but are not limited to, ear diseases, autoimmune ear diseases (Yoo TJ. *et al.*, Cell Immunol. 1994. 157(1):249) and autoimmune diseases of the inner ear (Gloddek B. *et al.*, Ann N Y Acad Sci. 1997. 830:266).

20 Examples of autoimmune systemic diseases include, but are not limited to, systemic lupus erythematosus (Erikson J. *et al.*, Immunol Res. 1998. 17(1-2):49) and systemic sclerosis (Renaudineau Y. *et al.*, Clin Diagn Lab Immunol. 1999. 6(2):156); Chan OT. *et al.*, Immunol Rev. 1999. 16:107).

Examples of infectious diseases include, but are not limited to, chronic infectious diseases, subacute infectious diseases, acute infectious diseases, viral diseases, bacterial diseases, protozoan diseases, parasitic diseases, fungal diseases, mycoplasma diseases and prion diseases.

25 Examples of transplantation-related diseases include, but are not limited to, graft rejection, chronic graft rejection, subacute graft rejection, hyperacute graft rejection, acute graft rejection and graft-versus-host disease.

Examples of grafts include syngeneic grafts, allografts, xenografts, cellular grafts, tissue grafts, organ grafts and appendage grafts.

30 Examples of cellular grafts include, but are not limited to, stem cell grafts, progenitor cell grafts, hematopoietic cell grafts, embryonic cell grafts and a nerve cell grafts.

Examples of tissue grafts include, but are not limited to, skin grafts, bone grafts, nerve grafts, intestine grafts, corneal grafts, cartilage grafts, cardiac tissue grafts, cardiac valve grafts, dental grafts, hair follicle grafts and muscle grafts.

5 Examples of organ grafts include, but are not limited to, kidney grafts, heart grafts, skin grafts, liver grafts, pancreatic grafts, lung grafts and intestine grafts.

Examples of appendage grafts include, but are not limited to, arm grafts, leg grafts, hand grafts, foot grafts, finger grafts, toe grafts and sexual organ grafts.

10 Examples of inflammatory diseases include, but are not limited to; injuries, neurodegenerative diseases, ulcers, inflammation associated with prosthetic implants, menstruation, septic shock, anaphylactic shock, toxic shock syndrome, cachexia, necrosis, gangrene, musculo-skeletal inflammation and idiopathic inflammation.

15 Examples of prosthetic implants include, but are not limited to, breast implants, silicone implants, dental implants, penile implants, cardiac implants, artificial joints, bone fracture repair devices, bone replacement implants, drug delivery implants, catheters, pacemakers, respirator tubes and stents.

Examples of ulcers include, but are not limited to, skin ulcers, bed sores, gastric ulcers, peptic ulcers, buccal ulcers, nasopharyngeal ulcers, esophageal ulcers, duodenal ulcers, ulcerative colitis and gastrointestinal ulcers.

20 Examples of injuries include, but are not limited to, abrasions, bruises, cuts, puncture wounds, lacerations, impact wounds, concussions, contusions, thermal burns, frostbite, chemical burns, sunburns, dessications, radiation burns, radioactivity burns, smoke inhalation, torn muscles, pulled muscles, torn tendons, pulled tendons, pulled ligaments, torn ligaments, hyperextensions, torn cartilage, bone fractures, pinched nerves and a gunshot wounds.

25 Examples of musculo-skeletal inflammations include, but are not limited to, muscle inflammations, myositis, tendon inflammations, tendinitis, ligament inflammations, cartilage inflammation, joint inflammations, synovial inflammations, carpal tunnel syndrome and bone inflammations.

As used herein the term "about" refers to  $\pm 10$  percent.

30 It is expected that during the life of this patent many relevant medical diagnostic techniques will be developed and the scope of the term "detecting" when relating to the target antigen is intended to include all such new technologies *a priori*.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

### EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.*, (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.*, (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984);

"Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak *et al.*,  
5 "Strategies for Protein Purification and Characterization – A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

10 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

15  
**EXAMPLE 1**  
**Generation of antibodies capable of binding NIK phosphorylated at aminoacid T559**

As described above, no treatment, or no satisfactory treatment, is available for  
20 numerous diseases associated with deregulated NF- $\kappa$ B activity, including malignant diseases and diseases associated with pathological immune responses, such as autoimmune, allergic, inflammatory, transplantation-related diseases. Since NIK is a critical activator of NF- $\kappa$ B, one potentially potent strategy for treating such diseases involves identifying antibodies capable of specifically binding NIK, and of thereby  
25 preventing or inhibiting activation of NF- $\kappa$ B by NIK. Such antibodies would also have utility as detection reagents enabling characterization of normal and pathological aspects of biological and biochemical processes involving NIK. The prior art, however, has failed to provide antibodies suitable or optimally suitable for such purposes. It has been reported that phosphorylation of aminoacid T559 located in the  
30 activation loop of NIK is important for its activity. A suitable antibody with such specificity has not been reported.

Thus a candidate antibody for the inhibition of NIK is that recognizing NIK

phosphorylated at amino acid residue T559 or fragments thereof.

In order to generate polyclonal antibodies specific for phosphorylated NIK, each of the overlapping set of NIK-activation loop derived peptides shown in Table 1 all containing the phospho threonine at position 559 (Fig 1) were used to immunize groups of rabbits.

**Table 1. activation loop and peptides used for immunizations.**

Peptide designation*	Amino acid sequence
553-566	CGDYI PG T(p)E THMAPE
553-562	KG DYI PG T(p)E TH
549-560	CSLLTG DYI PG T(p)E
534-566 Activation loop	DFGHAVCLQPDGLGK SLLTG DYI PG T E THMAPE

- numbers correspond to amino acid coordinates of peptide within NIK sequence.
- Note that to peptide 549-560 and 553-566 a cysteine has been added at the N-terminal end and to peptide 553-562 a lysine has been added to the N-terminal end.

It was observed by ELISA (Example 3), using phospho and non phospho peptides coated microtiter plates that the phosphorylated peptide set forth at SEQ ID NO: 3 out of the 3 activation loop derived peptides tested the peptide set forth in SEQ ID 3, in which the phosphorylated threonine is in the penultimate amino acid, was the only effective peptide in eliciting antibodies specific to the phosphorylated version of the peptide.

In view of the results obtained with polyclonal antibodies, monoclonal antibodies were prepared using the phosphorylated peptide set forth in SEQ ID N:3. (549-560) for immunization of Balb/C mice. Three fusions were carried out using spleen and lymph node of Balb/C immunized mice, however the resultant antibodies were non-specific or had low titer (tested by ELISA).

When instead of Balb/C mice, SJL immunized mice were used for the fusions, about 24 clones producing antibodies highly specific for T(p) 559 NIK peptide (tested by ELISA or Western blot) were generated.



## Example 2

### Monitoring specificity of antibodies by immunoprecipitation.

Further experiments were carried out to check whether the antibodies prepared against the 549-560 phosphorylated peptide are capable of recognizing the entire NIK protein and whether they are capable of differentiate the phosphorylation status of the protein. The experimental setting used include overexpression of myc tagged NIK (Example 11) or a myc tagged NIK mutant, having threonine at position 559 substituted to alanine in 293T cells. Following NIK overexpression the cells were lysed and NIK or NIK mutant, unable to get phosphorylated, were immunoprecipitated (IP) with the monoclonal antibodies of clones 869, 815, 521, 254, 91, 62, 32, 30 or 3, prepared with phosphorylated 549-560 peptide. Immunoprecipitated NIK was detected in Western blots employing anti-myc antibody.

More specifically, for overexpression of NIK,  $3 \times 10^6$  293T cells were seeded in a 15 cm plate and the cells were transiently transfected with pCS3MTNIK or pCS3MTNIKT559A. 24 hours later, the cells were harvested and lysed in 2ml 1% NP-40 buffer. 100ul of the lysate was used for each IP. IP was carried out by contacting the cell lysate with 30ul of protein G beads adsorbed with the respective antibodies for 4 hours at 4°C.

Western blotting and positive control for IP was performed with anti-myc antibody.

Anti-mic antibody detected both, phosphorylated and nonphosphorylated forms of NIK immunoprecipitated with the above antibodies. The results in Fig 4 show that all the tested antibodies were able to immunoprecipitate NIK. Clones 869, 815, 521, 254, 91, 62, 32, 30 and 3 were found to be specific for the phosphorylated form of NIK.

The results therefore indicate that thanks to the procedure employed, it was possible to generate antibodies specific for the phosphorylated species of NIK.

## Example 3

### Monitoring the specificity of NIK-P4 30.12 antibody by ELISA

Control non phosphorylated 549-560 peptide or phosphorylated type, at a

concentration of 10ug/ml, were used to coat wells of a microtiter plate. Undiluted hybridoma culture supernatant of clone NIK-P4 30.12 was loaded for 1 hour at 37°C to each of the coated wells. Anti mouse HRP (Jackson) at 1:10K dilution was used as the secondary antibody and O.D 405 was measured using ABTS substrate.

- 5 The results observed (Fig 5) demonstrate that the antibodies NIK-P4 30.12 recognise only the phosphorylated form of the peptide by ELISA.

#### Example 4

10

#### **Demonstration of inhibitory activity of NIK-P4 30.12 antibody in signalling through CD40 and CD70.**

15 The ligands of the TNF receptor CD70, CD40 and TNF induce I $\kappa$ B degradation and consequently induce NF $\kappa$ -B activation. To check whether NIK-P4 30.12 is able to inhibit the activity of NIK, the effect of NIK-P4 30.12 antibody on ligand-induced degradation of I $\kappa$ B was tested.

20 About  $0.5 \times 10^6$  Ramos cells were plated in 6 well plates and transfected with NIK-P4 30.12 (  $\alpha$ -pNIK) or a control non-relevant IgG (Example 12) and after transfection were induced with 1:4 dilution of the CD70 or CD40 ligand containing medium (see preparation of the ligand containing medium in Example 13) ) for 20 min and 30min respectively or with TNF used at a concentration of 50ng/ml for 20 min. or remained untreated. After the induction with the ligand, the cells were lysed in 45  $\mu$ l of 1% NP40 buffer + 15  $\mu$ l of sample loading buffer and 30  $\mu$ l of the lysate was loaded in a  
25 10% SDS-PAGE and subjected to Western blotting employing for the detection of the I $\kappa$ B anti I $\kappa$ B antibody (purchased from Transduction laboratories).

30 It was observed (Fig. 6) that in non treated cells (control) the I $\kappa$ B was present in both cells transfected with IgG or with anti-pNIK. However, in all ligand treated cells the I $\kappa$ B become degraded indicating activation of NF $\kappa$ -B. The I $\kappa$ B degradation and consequently NF $\kappa$ -B activation does not occur in cells transfected with the NIK-P4 30.12 antibodies except when the ligand employed is TNF. This results indicate that NIK phosphorylation at residue T559 is important for the activation of NF $\kappa$ -B by CD70 and CD40 and therefore NIK-P4 30.12 antibody can be used for therapy in diseases in which CD70 and CD40 signalling is involved in the pathogenesis of the  
35 disease.

**Example 5****Anti-phosphoThr559-NIK antibody blocks CD40 induced I $\kappa$ B degradation**

The following experiment was carried out in order to confirm that the inhibition of  
5 NIK activation observed with NIK-P4 30.12 does not occur with antibodies raised  
against domains in the kinase.

About  $0.5 \times 10^6$  BJAB cells were transfected with either non-specific IgG, antibody  
specific to the N terminal region of the kinase domain of NIK (specific for residues  
10 405-420) (81), or with NIK-P4 30.12. All the transfected cells were treated with  
CD40L as in the previous example for 30min or remained untreated. Following the  
treatment, the cells were lysed in 1% NP40 buffer and the I $\kappa$ B $\alpha$  in the cell was  
monitored by Western blot as in the preceding example.

15 It was observed (Fig 7) that CD40 induces degradation of I $\kappa$ B $\alpha$  in CD40 treated cells.  
Inhibition of I $\kappa$ B $\alpha$  degradation was not observed using very efficient antibodies to a  
different region of the kinase domain (81) and the only antibody that inhibited  
degradation of the I $\kappa$ B $\alpha$  was NIK-P4 30.12. This result indicate that the NIK-P4 30.12  
20 antibodies raised to the phosphorylated T559 in the activation loop of NIK inhibit the  
degradation of I $\kappa$ B and consequently NF $\kappa$ -B activation by CD40L treatment.

**Example 6****Preparation of polyclonal antibody**

Rabbits were immunized with peptides set forth in SEQ ID NO:1, 2 or 3 for the  
25 generation of specific polyclonal antibodies.

The first immunization was carried out with 100 $\mu$ g of peptide-KLH which was  
dissolved in 1:1 complete Freund's Adjuvant and injected sub-cutaneously. A second  
immunization was carried out three weeks later with the same amount of peptide-KLH  
and injected intramuscularly three weeks later with incomplete Freund's adjuvant.

30 These two immunizations were followed one boost of the same amount of peptide-  
KLH dissolved in PBS and administered sub-cutaneously at three weeks interval.

**Example 7:**

### **Preparation of monoclonal antibodies**

Monoclonal antibodies were prepared as described by Eshhar Z. in: "Hybridoma Technology in the Biosciences and Medicine", Chapter 1, Springer TA. (eds.) Timothy A., Plenum Publishing Corp., New York (1985)] using spleen of the positive mice challenged with peptide of SEQ ID NOs: 7, 11 and 12 of the kinase N and C- terminus for fusion of spleen and lymph nodes.

Culture supernatants of the hybridomas were tested by western blotting to detect myc tagged NIK.

1.5 X 10<sup>6</sup> 293-T cells were seeded on 10cm plates and transfected after 24 hours with pCS3MTNIK . A 24 h later the transfected cells were harvested and lysed in 1 ml 1%NP40 lysis buffer. 40 µl of the lysate was loaded per lane along with pcDNA3 transfected cell lysate as a control (C). Western blots were probed with the respective hybridoma culture supernatant.

### **Example 8:**

#### **Immunoprecipitation with immune serum:**

For immunoprecipitation of recombinant NIK fusion protein from transfectant protein lysate using immune serum, 1.5 µl of immune serum was mixed with 25 µl of protein G-conjugated beads and 50 µl of lysate, and the volume was completed to 750 µl with lysis buffer. The mixture was incubated at 4 °C for 2 hours. Following incubation, the beads were washed three times with lysis buffer, boiled in 30 µl of sample buffer, and microcentrifuged at 15,000 rpm for 2 minutes. The supernatant (immunoprecipitate) was collected and analyzed for the presence of human NIK via 10 % SDS-PAGE.

#### **Immunoprecipitation with sup from hybridoma:**

293-T lysate expressing myc tagged NIK protein was used as substrate for testing the immunoprecipitating ability of the monoclonal antiNIK antibodies. 50 µl of protein G beads was mixed with 500ul of hybridoma culture supernatant and incubated at about 22°C for 1 hour. Bead were washed with 1% NP-40 lysis buffer three times and used for immunoprecipitation.

Immunoprecipitation (IP) was carried out for 2 hours at +4 °C. After IP, the beads

were washed three times and boiled with 50 µl of Laemli buffer and 25µl supernatant was loaded on 10% SDS-PAGE.

## 5 Example 9

### Western immunoblotting analysis:

For 20 wells slot blot, multi-screen Biorad, a 180 µl aliquot of lysate from PCS3MTNIK –transfected 293-T cells was analyzed via SDS-PAGE preparative gel.

10 As a positive control, anti-myc antibody was used at 1:1,000 dilution.

## Example 10

### ELISA:

Lysate from pcHis-NIK–transfected cells was diluted 25-fold dilution in binding  
15 buffer, and 50 µl/well was used for coating ELISA plate wells. Detection of coated  
NIK or BSA coupled peptide was performed using a 1:100 dilution of anti-NIK  
immune serum, and the assay was developed using HRP-conjugated sheep anti-mouse  
antibody with ABTS as enzyme substrate. The OD<sub>405</sub> of the samples was determined  
after a 20-minute reaction time.

20

## Example 11

### 25 Production of recombinant human NIK:

For expression of recombinant human NIK fused to a myc (myc-NIK) or polyhistidine  
(His-NIK) affinity tag,  $3 \times 10^6$ , or  $1.5 \times 10^6$  293-T cells, respectively, were transfected  
with expression vector PCS3MTNIK encoding myc tagged NIK or pcHis-NIK  
encoding His tagged NIK, respectively. Cells were transfected via the calcium  
30 phosphate  $[Ca_3(PO_4)_2]$  method in 10 cm-diameter culture dish using 20 µg or 10 µg of  
expression vector DNA. Twenty-four hours following transfection, PCS3MTNIK or  
pcHis-NIK transfected cells were harvested, pelleted and lysed in 1.5 ml or 1 ml,  
respectively, of 1 % NP-40 protein lysis buffer.

### Example 12

#### Transfection of antibody in the cell:

5

In order to test the effect of the antibodies inside the cells, the cells were transfected with the antibody employing the project reagent of Pierce using the following protocol.

$2 \times 10^5$  cells were seeded in a 6-well plate and cultured grow overnight.

10

10  $\mu$ l of Pro-Ject<sup>TM</sup> Reagent (dissolved in 250  $\mu$ l of methanol or chloroform) were used and evaporated under a laminar flow hood for 2 hours. 5-10  $\mu$ g of protein, the FITC-Ab control or tested monoclonal antibody were diluted in 50-100  $\mu$ l.

15

The dried Pro-Ject<sup>TM</sup> film was hydrated with the in 50-100  $\mu$ l of the diluted protein solution by pipetteing up and down about 3 to 5 times and incubate at room temperature for 5 minutes and Vortex for a few seconds at low to medium speed.

The final volume of the Pro-Ject<sup>TM</sup> Reagent/protein mixture was brought up to 1,000  $\mu$ l with serum free medium.

20

The medium from the cells to be tested was removed by centrifugation, washed one time with serum-free medium and the Pro-Ject<sup>TM</sup> Reagent/protein mixture was transferred directly onto the cells.

### Example 13

#### Preparation of medium containing CD70 or CD40 used for induction:

25

30

293T cells were transfected with a Flag-tagged leucine-zippered CD40/CD70 construct (Walczak et al. 1999, Fanslow et al. 1994 ) molecule. In this molecule the ligand is already multimerized by virtue of the leucine-zippered fused to it. DNA encoding for Flag-tagged Leucine-zippered ligand was cloned into the pcDNA mammalian expression vector (Invitrogen) and transiently expressed in HEK-293 cells. The supernatant of these cells was harvested three days post transfection, filter sterilized and used.

35

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such  
5 alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents, and patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if  
10 each individual publication, patent, or patent application or sequence identified by its accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

- 15 **Hybridoma clone Nik-P4 30.12 was deposited at the Collection Nationale de Culture de Microorganismes (CNCM), Institut Pasteur, Paris, under the Budapest Treaty and were accorded deposit No. I-3095. The hybridomas were registered at the CNCM at October 2, 2003.**

## Claims

1. An antibody or fragment thereof being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprises a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5.
2. An antibody or fragment of claim 1, wherein the portion comprises the amino acid sequence of SEQ ID NO:6.
3. An antibody or fragment of claim 1, wherein the portion comprises the amino acid sequence of SEQ ID NO:3.
4. The antibody of claim 1, wherein said antibody is an IgG antibody.
5. The antibody preparation of claim 1, wherein said antibody fragment is selected from the group consisting of a single-chain Fv, an Fab, an Fab', an F(ab')<sub>2</sub> and a CDR.
6. The antibody of claim 1, wherein said antibody or antibody fragment is further capable of regulating a biochemical activity of a NIK molecule.
7. The antibody according to anyone of the preceding claims, wherein said antibody or antibody fragment is further capable of specifically detecting phosphorylated NIK or a specific portion thereof.
8. The antibody according to claim 7 capable of specifically detecting phosphorylated NIK by Western blot analysis.
9. The antibody according to claim 7 capable of specifically detecting phosphorylated NIK by ELISA.
10. The antibody according to claim 7 capable of specifically detecting phosphorylated NIK by immunoprecipitation.
11. A monoclonal antibody or fragment thereof being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5.



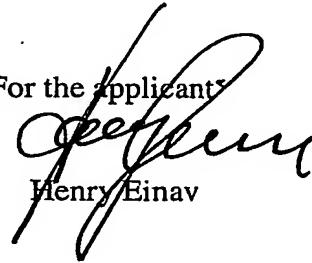
12. The monoclonal antibody of claim 11, being capable of specifically binding the amino acid sequence set forth in SEQ ID NO:3.
13. The monoclonal antibody of claim 11, being monoclonal antibodies generated by hybridoma NIK-P4 30.12 deposited at the CNCM under No. I-3095.
14. An hybridoma clone deposited at the CNCM under No. I-3095
15. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active ingredient, being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5.
16. The pharmaceutical composition of claim 15, wherein the amino acid portion comprises the amino acid sequence set forth in SEQ NO: 3.
17. The pharmaceutical composition of claim 15, wherein said antibody is an IgG antibody.
18. The pharmaceutical composition of claim 15, wherein said antibody or antibody fragment is derived from mouse.
19. The pharmaceutical composition of claim 15, wherein said antibody fragment is selected from the group consisting of a single-chain Fv, an Fab, an Fab', and F(ab')<sub>2</sub> and a CDR.
20. The pharmaceutical composition of claim 15, wherein said antibody or antibody fragment is further capable of regulating a biochemical activity of a NIK molecule.
21. The pharmaceutical composition of claim 20 for the inhibition of CD40 signalling.
22. The pharmaceutical composition of claim 20 for the inhibition of CD70 signalling.

23. A method of regulating a biochemical activity of a NIK molecule, the method comprising contacting the NIK molecule with being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5, thereby regulating a biochemical activity of a NIK molecule.
24. The method of claim 23, wherein said contacting the NIK molecule with said preparation is effected by administering said preparation to an individual.
25. The method of claim 23, wherein said antibody is an IgG antibody.
26. The method of claim 23, wherein said antibody or antibody fragment is derived from mouse.
27. The method of claim 23, wherein said antibody fragment is selected from the group consisting of a single-chain Fv, an Fab, an Fab', an F(ab')<sub>2</sub> and a CDR.
28. A composition-of-matter comprising a substrate covalently attached to a peptide of amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 for selectively capturing the antibody or antibody fragment capable of specifically binding the target antigen.
29. The composition-of-matter of claim 28, wherein the portion thereof comprises the amino acid sequence set forth in SEQ ID NO:3.
30. The composition-of-matter of claim 28, wherein said substrate is an affinity chromatography matrix.
31. The composition-of-matter of claim 28, wherein said substrate comprises a carbohydrate or a derivative of said carbohydrate.

32. The composition-of-matter of claim 28, wherein said carbohydrate is selected from the group consisting of agarose, sepharose, and cellulose.
33. The composition-of-matter of claim 28, wherein said substrate is selected from the group consisting of a bead, a resin, or a plastic surface.
34. A method of treatment of a disease caused or aggravated by the activity of NIK, comprising the administration of an antibody being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 to an individual in need.
35. The method of claim 34, wherein said antibody is an IgG antibody.
36. The method of claim 34, wherein said antibody or antibody fragment is derived from mouse.
37. The method of claim 34, wherein said antibody fragment is selected from the group consisting of a single-chain Fv, an Fab, an Fab', an F(ab')<sub>2</sub> and a CDR.
38. The use of an antibody or antibody fragment being capable of specifically binding the amino acid sequence set forth in SEQ ID NO: 5, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 in the manufacture of a medicament for the treatment of a disease caused or aggravated by the activity of NIK.
39. The use of claim 38, wherein said antibody is an IgG antibody.
40. The use of claim 38, wherein said antibody or antibody fragment is derived from mouse.
41. The use of claim 38, wherein said antibody fragment is selected from the group consisting of a single-chain Fv, an Fab, an Fab', an F(ab')<sub>2</sub> and a CDR.

42. A method for preparing a monoclonal antibody comprising growing a cloned hybridoma comprising a spleen cell from a mammal immunized with an amino acid sequence comprising SEQ ID NO: 6, or a portion thereof wherein the amino acid sequence or portion thereof comprising a phosphorylated threonine at amino acid position 559 of SEQ ID NO: 5 and a homogeneous or heterogeneous lymphoid cell in liquid medium or mammalian abdomen to allow the hybridoma to produce and accumulate the monoclonal antibody.
43. A method of claim 42, wherein the portion thereof comprises amino acid sequence set forth in SEQ ID NO: 3.

For the applicant:

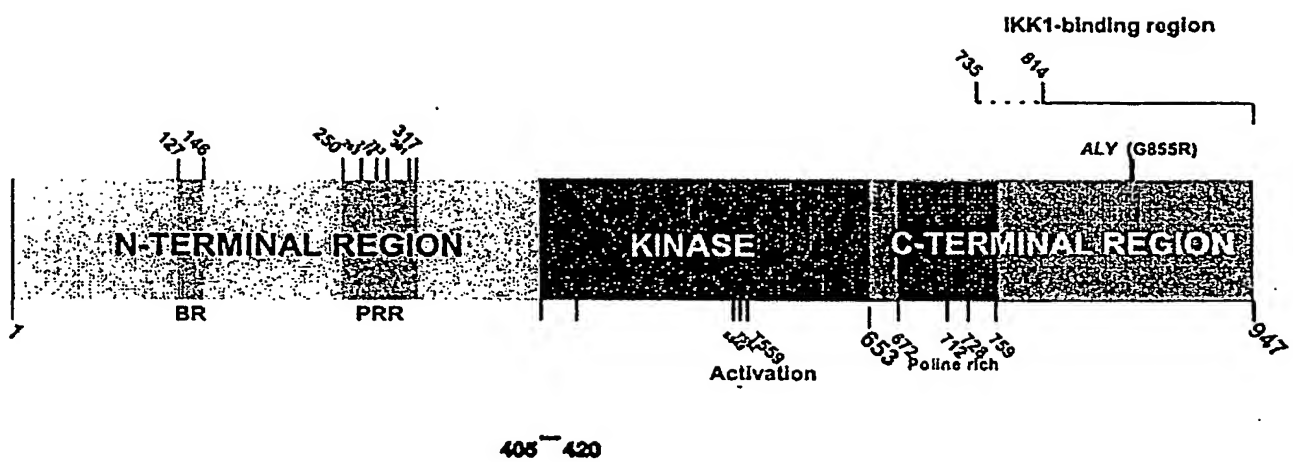


Henry Einav

**ABSTRACT OF THE DISCLOSURE**

An antibody or antibody fragment preparation being capable of specifically binding the amino acid sequence 60-76, 86-98, 135-149, 215-227, 363-377, 385-397, 405-419, 427-440, 509-522, 605-619, 635-649, 666-680, 696-711, 752-766, 836-851, 871-884, and/or 904-916 of NIK, or being capable of specifically binding a specific portion of the amino acid sequence is provided.

**Figure 1**



**Figure 2**

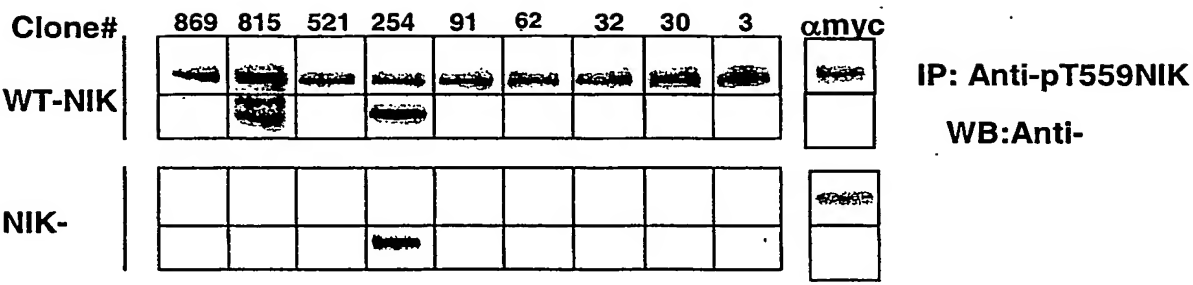
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301	LSKLACVDSP	KPLPGPHLEP	SCLSRGAHEK	FSVEEYLVHA	LQGSVSSSQA	350
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**Figure 3**

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Figure 4



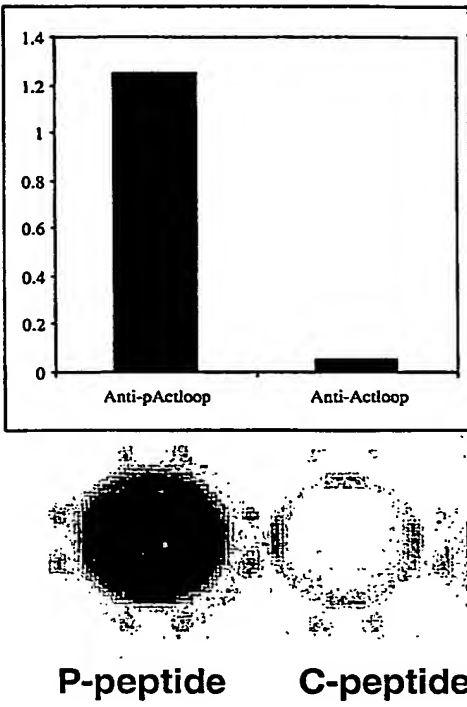


Figure 5

Figure 6

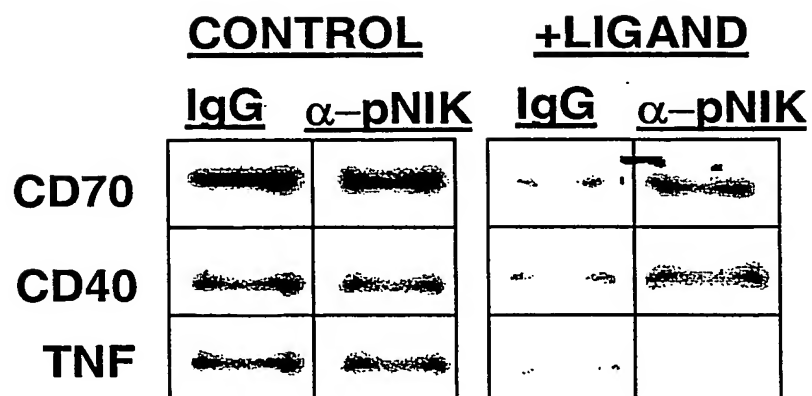
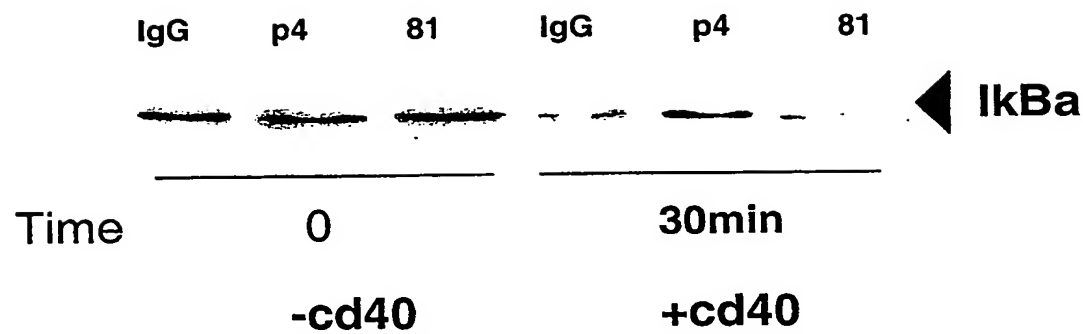


Figure 7



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